REMARKS

Applicants have carefully studied the Office Action mailed on February 26, 2008, which issued in connection with the above-identified application. The present amendments and remarks are intended to be fully responsive to all points of rejection raised by the Examiner and are believed to place the claims in condition for allowance. Favorable reconsideration and allowance of the present claims are respectfully requested.

Pending Claims

Claims 18-34 were pending and at issue in the application. Claims 26-27 and 32-33 have been rejected under 35 U.S.C. §112, first paragraph, for lack of written description. Claims 18-25, 28-31, and 34 have been rejected under 35 U.S.C. §103(a) as being obvious over WO 97/44015. Claims 18-25, 28-31, and 34 have been also rejected on the ground of non-statutory obviousness-type double patenting as being unpatentable over claims 1-4 and 13 of the U.S. Patent No. 6,596,318.

To expedite prosecution (and not as an admission of correctness of the Examiner's rejections), claims 26-28 and 32-33 have been canceled without prejudice or disclaimer. Applicants preserve the right to prosecute the subject matter of the cancelled claims in a continuing application. Claim 18 has been amended to introduce the limitations of canceled claim 28. Specifically, claim 18 has been amended to recite that an active ingredient is "selected from the group consisting of antibiotics, corticosteroids, antimycotics, neuroleptics, antiepileptics, steroid hormones, anticancer hormones, antirheumatics, and combinations thereof."

The aforementioned amendments do not add new matter to the application. As a result of the amendments, claims 18-25, 29-31, and 34 are now pending.

Response to Rejections

The rejections set forth in the Office Action are summarized and addressed as follows.

(1) Rejection under 35 U.S.C. §112, first paragraph (written description)

Claims 26, 27, 32, and 33 have been rejected for lack of written description for recitation of a medicament formulation comprising ceramic granules or calcium phosphate (claims 27 and 27) or for recitation of a bone replacement implant (claims 32 and 33). The Examiner's position is that the specification does not teach how to make such a medicament formulation or bone replacement implant and no examples have been provided.

To expedite prosecution (and not as an admission of correctness of the Examiner's rejections), claims 26, 27, 32, and 33 have been canceled without prejudice or disclaimer. The rejection of these claims for lack of written description is therefore rendered moot.

(2) Obviousness Rejection under 35 U.S.C. §103(a)

In the Office Action, claims 18-25, 28-31, and 34 have been rejected under 35 U.S.C. §103(a) as being obvious over WO 97/44015 (Heath et al.). The Examiner argues that, while WO 97/44015 does not disclose the fluidized bed drying step of claim 18, the process of fluidized bed drying is not essential to a determination of the patentability of the claimed formulation, because the determination of the patentability of a product-by-process claim is based on the product itself (and does not depend on its method of production) and such claim may be obvious over a product made by a different process. The Examiner concludes that one of ordinary skill in the art would be motivated to make the formulation of the present claims based on the disclosure of WO 97/44015, because WO 97/44015 discloses "good flow properties, enhanced, effective delivery to the active site, and dissolution only at the site." In addition, the Examiner notes that the 20-500 μm particle size recited in the present claims cannot be used as a distinguishing characteristic, since WO 97/44015 discloses particles of up to 50 μm at p. 3, l. 14.

Since claim 28 has been canceled, the rejection of this claim is rendered moot.

As specified above, to expedite prosecution (and not as an admission of correctness of the Examiner's rejections), claim 18 has been amended to recite that an active ingredient is "selected from the group consisting of antibiotics, corticosteroids, antimycotics, neuroleptics, antiepileptics, steroid hormones, anticancer hormones, antirheumatics, and combinations thereof." All other claims depend from claim 18.

The amendment to claim 18 emphasizes that the key point of the present invention is a provision of a biodegradable carrier consisting of blood plasma protein(s) wherein said carrier is able to immobilize active ingredients such as antibiotics, corticosteroids, antimycotics, neuroleptics, antiepileptics, steroid hormones, anticancer hormones, antirheumatics, and combinations thereof.

In contrast to the present claims, WO 97/44015 does not disclose or suggest the possibility of immobilizing active ingredients such as antibiotics, corticosteroids, antimycotics, neuroleptics, antiepileptics, steroid hormones, anticancer hormones, antirheumatics, and combinations thereof in the microparticles. WO 97/44015 merely describes the production of microparticles based on blood plasma proteins.

Also, as acknowledged by the Examiner, the microparticles disclosed in WO 97/44015 are produced by *spray-drying*, while the microparticles recited in the present claims are produced by *fluidized bed drying*. In contrast to the Examiner's assertion, applicants respectfully note that the production process constitutes a limitation of the present product claims, because the process of fluidized bed drying of the present invention produces microparticle formulations which are *materially different in their physico-chemical properties* as compared to the formulations of WO 97/44015 produced using spray drying. The larger average size of the microparticles recited in the present claims is only *one of several inherent characteristics* of these particles that distinguish them from the microparticles of WO 97/44015. As discussed in the Rule 132 Declaration of Prof. Schmidt which has been presented to the Examiner on April 5, 2007, the distinguishing characteristics between microparticles of the present invention and microparticles of WO 97/44015 include particle morphology, stability, solubility, dusting and handling properties. For example, the microparticles of the present invention are compact, slightly porous solid particles not having hollow-sphere characteristics. In contrast, the microparticles of WO 97/44015 are hollow

microspheres. As recited at p. 3, ll. 9-11 of WO 97/44015, "Microparticles comprising fibrinogen or thrombin may be prepared by the procedures described in WO-A-9218164, WO-A-9609814 and WO-A-9618388." International Publication Nos. WO-A-9218164, WO-A-9609814 and WO-A-9618388 (attached as Exhibits A-C, respectively) disclose production of microparticles useful for ultrasound imaging for which being *hollow* is a highly desirable feature. As specified at p. 13, ll. 29-32 of WO-A-9618388, "The microparticles were *hollow* and contained air which enabled their passage and persistence in the blood stream to be followed using ultrasound imaging" (emphasis added). As further specified at p. 3, ll. 30-31 of WO-A-9609814, "Unless the particles are *hollow*, they are unsuitable for echocardiography" (emphasis added). See also p. 8, l. 35 - p. 9, l. 17 of WO-A-9609814 and p. 13, l. 34 - p. 15, l. 20 of WO-A-9218164.

Taken together, as a result of being produced by fluidized bed drying, the microparticles recited in the present claims have several inherent physico-chemical properties which distinguish them from the spray-dried microparticles disclosed in WO 97/44015. By specifying that the product recited in the present claims is produced by fluidized bed drying, the need for reciting each of its distinguishing inherent physico-chemical properties is avoided.

In light of the above arguments, it is believed that the resent claims are not obvious over WO 97/44015. Withdrawal of the obviousness rejection is respectfully requested.

(3) Double Patenting Rejection.

In the Office Action, claims 18-25, 28-31, and 34 have been also rejected on the ground of non-statutory obviousness-type double patenting as being unpatentable over claims 1-4 and 13 of the U.S. Patent No. 6,596,318 ('318 patent). The Examiner contends that, although the conflicting claims are not identical, they are not patentably distinct from each other, because the '318 patent claims a granulated blood plasma protein medicament formulation produced by fluidized bed drying. The Examiner concludes that, although the '318 patent does not claim an active ingredient, the use of active ingredients was known in the art at the time of the present invention.

In order to expedite the prosecution and without admitting the correctness of the double patenting rejection, applicants note that a Terminal Disclaimer will be filed upon the determination of

the allowable subject matter to disclaim any terminal part of the term of a patent which will issue on the present application after the '318 patent expires.

Applicants further note that, prior to filing the Terminal Disclaimer, Mirna Rapp will be added as a co-inventor and CSL Behring GmbH as an assignee of the present application. All documents essential for changing inventorship are currently being executed and will be submitted to the USPTO shortly.

CONCLUSION

Applicants request entry of the foregoing amendments and remarks in the file history of this application. In view of the above amendments and remarks, it is respectfully submitted that claims 18-25, 29-31, and 34 are now in condition for allowance and such action is earnestly solicited. If the Examiner believes that a telephone conversation would help advance the prosecution in this case, the Examiner is respectfully requested to call the undersigned attorney at (212) 527-7634. The Examiner is hereby authorized to charge any additional fees associated with this response to our Deposit Account No. 04-0100.

Dated: August 26, 2008

Respectfully submitted,

By _____

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EXHIBITA

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(54) Title: PREPARATION OF DIAGNOSTIC AGENTS

(57) Abstract

Microcapsules are prepared by a process comprising the steps of (i) spray-drying a solution or dispersion of a wall-forming material in order to obtain intermediate microcapsules and (ii) reducing the water-solubility of at least the outside of the intermediate microcapsules. Suitable wall-forming materials include proteins such as albumin and gelatin. The microcapsules have walls of 40-500 nm thick and are useful in ultrasonic imaging. The control of median size, size distribution and degree of insolubilisation and cross-linking of the wall-forming material allows novel microsphere preparations to be produced.

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PREPARATION OF DIAGNOSTIC AGENTS

The present invention relates to the preparation of diagnostic agents comprising hollow proteinaceous microcapsules used to enhance ultrasound imaging.

The fact that air bubbles in the body can be used for echocardiography has been known for some time. Bubble-containing liquids can be injected into the bloodstream for this purpose (see Ophir *et al* (1980) *"Ultrasonic Imaging"* 2, 67-77, who stabilised bubbles in a collagen membrane, US-A-4 446 442 (Schering) and EP-A-131 540 (Schering)) and EP-A-224934 and EP-A-324938 disclose the use of bubbles prepared by sonicating an albumin solution. However, the size distribution of the bubbles is apparently uncontrollable and the bubbles disappear when subjected to pressure experienced in the left ventricle (Shapiro *et al* (1990) *J. Am. Coll. Cardiology*, **16(7)**, 1603-1607).

EP-A-52575 discloses, for the same purpose, solid particles which have gas entrained in them, the gas being released from the particles in the bloodstream.

EP 458 745 (Sintetica) discloses a process of preparing air- or gas-filled microballoons by interfacial polymerisation of synthetic polymers such as polylactides and polyglycolides. WO 91/12823 (Delta) discloses a similar process using albumin. Wheatley *et al* (1990)

Biomaterials 11, 713-717 discloses ionotropic gelation of alginate to form microbubbles of over 30 μm diameter. WO 91/09629 discloses liposomes for use as ultrasound contrast agents.

We have now found that a process of atomising a solution of microcapsule-forming agent and then insolubilising the microcapsules which are formed leads to an improved product. Przyborowski *et al* (1982 *Eur. J. Nucl. Med.* 7, 71-72) disclosed the preparation of human serum albumin (HSA) microspheres by spray-drying for radiolabelling and subsequent use in scintigraphic imaging of the lung. The microspheres were not said to be hollow and, in our repetition of the work, only solid microspheres are produced. Unless the particles are hollow, they are unsuitable for echocardiography. Furthermore, the microspheres were prepared in a one step process which we have found to be unsuitable for preparing microcapsules suitable for echocardiography; it was necessary in the prior process to remove undenatured albumin from the microspheres (which is not necessary in our process); and a wide size range of microspheres was apparently obtained, as a further sieving step was necessary. Hence, not only was the Przyborowski *et al* process not an obvious one to choose for the preparation of microcapsules useful in ultrasonic imaging but the particles produced were unsuitable for that purpose. We have devised a considerable improvement over that prior process.

The Przyborowski *et al* article refers to two earlier disclosures of methods of obtaining albumin particles for lung scintigraphy. Aldrich & Johnston (1974) *Int. J. Appl. Rad. Isot.* **25**, 15-18 disclosed the use of a spinning disc to generate 3-70 μm diameter particles which are then denatured in hot oil. The oil is removed and the particles labelled with radioisotopes. Raju *et al* (1978) *Isotopenpraxis* **14(2)**, 57-61 used the same spinning disc technique but denatured the albumin by simply heating the particles. In neither case were hollow microcapsules mentioned and the particles prepared were not suitable for echocardiography.

One aspect of the present invention provides a process comprising a first step of atomising a solution or dispersion of a wall-forming material in order to obtain microcapsules.

Preferably, the product obtained thereby is subjected to a second step of reducing the water-solubility of at least the outside of the said microcapsules.

The said two steps may be carried out as a single process or the intermediate product of the first step may be collected and separately treated in the second step. These two possibilities are referred to hereinafter as the one step and two step processes.

The wall-forming material and process conditions should be so chosen that the product is sufficiently non-toxic and non-immunogenic in the conditions of use, which will clearly depend on the dose administered and duration of treatment. The wall-forming material may be a starch derivative, a synthetic polymer such as tert-butyloxycarbonylmethyl polyglutamate (US Patent No 4 888 398) or a polysaccharide such as polydextrose.

Generally, the wall-forming material can be selected from most hydrophilic, biodegradable physiologically compatible polymers. Among such polymers one can cite polysaccharides of low water solubility, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones such as ε-caprolactone, δ-valerolactone, polypeptides, and proteins such as gelatin, collagen, globulins and albumins. Other suitable polymers include poly-(ortho)esters (see for instance US-A-4,093,709; US-A-4,131,648; US-A-4,138,344; US-A-4,180,646; polylactic and polyglycolic acid and their copolymers, for instance DEXON (see J. Heller (1980) *Biomaterials* **1**, 51; poly(DL-lactide-co-δ-caprolactone), poly(DL-lactide-co-δ-valerolactone), poly(DL-lactide-co-g-butyrolactone), polyalkylcyanoacrylates; polyamides, polyhydroxybutyrate; polydioxanone; poly-β-aminoketones (*Polymer* **23** (1982), 1693); polyphosphazenes (*Science* **193** (1976), 1214); and polyanhydrides. References on biodegradable polymers can be found in R. Langer et *al* (1983) *Macromol. Chem. Phys.* **C23**, 61-125. Polyamino-acids

such as polyglutamic and polyaspartic acids can also be used as well as their derivatives, ie partial esters with lower alcohols or glycols. One useful example of such polymers is poly-(t,butyl-glutamate). Copolymers with other amino-acids such as methionine, leucine, valine, proline, glycine, alamine, etc are also possible. Recently some novel derivatives of polyglutamic and polyaspartic acid with controlled biodegradability have been reported (see WO 87/03891; US 4,888,398 and EP 130 935 incorporated here by reference). These polymers (and copolymers with other amino-acids) have formulae of the following type:

$$-(NH-CHA-CO)_x(NH-CHX-CO)_y$$

where X designates the side chain of an amino-acid residue and A is a group of formula $-(CH_2)_nCOOR^1R^2OCOR$(II), with R^1 and R^2 being H or lower alkyls, and R being alkyl or aryl; or R and R^1 are connected together by a substituted or unsubstituted linking member to provide 5- or 6-membered rings.

A can also represent groups of formulae:

$$-(CH_2)_nCOO-CHR^1COOR \qquad (I)$$

and

$$-(CH_2)_nCO(NH-CHX-CO)_mNH-CH(COOH)-(CH_2)_pCOOH \qquad (III)$$

and corresponding anhydrides. In all these formulae n, m and p are lower integers (not exceeding 5) and x and y are also integers selected for having molecular weights not below 5000.

The aforementioned polymers are suitable for making the microspheres according to the invention and, depending on the nature of substituents R, R^1, R^2 and X, the properties of the wall can be controlled, for instance, strength, elasticity and biodegradability. For instance X can be methyl (alanine), isopropyl (valine), isobutyl (leucine and isoleucine) or benzyl (phenylalanine).

Preferably, the wall-forming material is proteinaceous. For example, it may be collagen, gelatin or (serum) albumin, in each case preferably of human origin (ie derived from humans or corresponding in structure to the human protein). Most preferably, it is human serum albumin (HA) derived from blood donations or, ideally, from the fermentation of microorganisms (including cell lines) which have been transformed or transfected to express HA.

Techniques for expressing HA (which term includes analogues and fragments of human albumin, for example those of EP-A-322094, and polymers of monomeric albumin) are disclosed in, for example, EP-A-201239 and EP-A-286424. All references are included herein by reference. "Analogues and fragments" of HA include all polypeptides (i) which are capable of forming a microcapsule in the process of the invention and (ii) of which a continuous region of at least 50% (preferably at least 75%, 80%, 90% or 95%) of the amino acid sequence is at least 80% homologous (preferably at least 90%, 95% or 99% homologous) with a continuous region

of at least 50% (preferably 75%, 80%, 90% or 95%) of human albumin. HA which is produced by recombinant DNA techniques is particularly preferred. Thus, the HA may be produced by expressing an HA-encoding nucleotide sequence in yeast or in another microorganism and purifying the product, as is known in the art. Such material lacks the fatty acids associated with serum-derived material. Preferably, the HA is substantially free of fatty acids; ie it contains less than 1% of the fatty acid level of serum-derived material. Preferably, fatty acid is undetectable in the HA.

In the following description of preferred embodiments, the term "protein" is used since this is what we prefer but it is to be understood that other biocompatible wall-forming materials can be used, as discussed above.

The protein solution or dispersion is preferably 0.1 to 50% w/v, more preferably about 5.0 - 25.0% protein, particularly when the protein is albumin. About 20% is optimal. Mixtures of wall-forming materials may be used, in which case the percentages in the last two sentences refer to the total content of wall-forming material.

The preparation to be sprayed may contain substances other than the wall-forming material and solvent or carrier liquid. Thus, the aqueous phase may contain 1-20% by weight of water-soluble hydrophilic compounds like sugars

and polymers as stabilizers, eg polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), gelatin, polyglutamic acid and polysaccharides such as starch, dextran, agar, xanthan and the like. Similar aqueous phases can be used as the carrier liquid in which the final microsphere product is suspended before use. Emulsifiers may be used (0.1-5% by weight) including most physiologically acceptable emulsifiers, for instance egg lecithin or soya bean lecithin, or synthetic lecithins such as saturated synthetic lecithins, for example, dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline or distearoyl phosphatidyl choline or unsaturated synthetic lecithins, such as dioleyl phosphatidyl choline or dilinoleyl phosphatidyl choline. Emulsifiers also include surfactants such as free fatty acids, esters of fatty acids with polyoxyalkylene compounds like polyoxypropylene glycol and polyoxyethylene glycol; ethers of fatty alcohols with polyoxyalkylene glycols; esters of fatty acids with polyoxyalkylated sorbitan; soaps; glycerol-polyalkylene stearate; glycerol-polyoxyethylene ricinoleate; homo- and copolymers of polyalkylene glycols; polyethoxylated soya-oil and castor oil as well as hydrogenated derivatives; ethers and esters of sucrose or other carbohydrates with fatty acids, fatty alcohols, these being optionally polyoxyalkylated; mono-, di- and triglycerides of saturated or unsaturated fatty acids, glycerides or soya-oil and sucrose.

Additives can be incorporated into the wall of the microspheres to modify the physical properties such as dispersibility, elasticity and water permeability.

Among the useful additives, one may cite compounds which can "hydrophobize" the wall in order to decrease water permeability, such as fats, waxes and high molecular-weight hydrocarbons. Additives which improve dispersibility of the microspheres in the injectable liquid-carrier are amphipathic compounds like the phospholipids; they also increase water permeability and rate of biodegradability.

Additives which increase wall elasticity are the plasticizers like isopropyl myristate and the like. Also, very useful additives are constituted by polymers akin to that of the wall itself but with relatively low molecular weight. For instance when using copolymers of polylactic/polyglycolic type as the wall-forming material, the properties of the wall can be modified advantageously (enhanced softness and biodegradability) by incorporating, as additives, low molecular weight (1000 to 15,000 Dalton) polyglycolides or polylactides. Also polyethylene glycol of moderate to low Mw (eg PEG 2000) is a useful softening additive.

The quantity of additives to be incorporated in the wall is extremely variable and depends on the needs. In some cases no additive is used at all; in other cases amounts of

additives which may reach about 20% by weight of the wall are possible.

The protein solution or dispersion (preferably solution), referred to hereinafter as the "protein preparation", is atomised and spray-dried by any suitable technique which results in discrete microcapsules of 1.00 - 50.0 μm diameter. These figures refer to at least 90% of the population of microcapsules, the diameter being measured with a Coulter Master Sizer II. The term "microcapsules" means hollow particles enclosing a space, which space is filled with a gas or vapour but not with any solid materials. Honeycombed particles resembling the confectionery sold in the UK as "Maltesers" (Regd TM) are not formed. It is not necessary for the space to be totally enclosed (although this is preferred) and it is not necessary for the microcapsules to be precisely spherical, although they are generally spherical. If the microcapsules are not spherical, then the diameters referred to above relate to the diameter of a corresponding spherical microcapsule having the same mass and enclosing the same volume of hollow space as the non-spherical microcapsule.

The atomising comprises forming an aerosol of the protein preparation by, for example, forcing the preparation through at least one orifice under pressure into, or by using a centrifugal atomizer in a chamber of warm air or

other inert gas. The chamber should be big enough for the largest ejected drops not to strike the walls before drying. The gas or vapour in the chamber is clean (ie preferably sterile and pyrogen-free) and non-toxic when administered into the bloodstream in the amounts concomitant with administration of the microcapsules in echocardiography. The rate of evaporation of the liquid from the protein preparation should be sufficiently high to form hollow microcapsules but not so high as to burst the microcapsules. The rate of evaporation may be controlled by varying the gas flow rate, concentration of protein in the protein preparation, nature of liquid carrier, feed rate of the solution and, most importantly, the temperature of the gas encountered by the aerosol. With an albumin concentration of 15-25% in water, an inlet gas temperature of at least about 100°C, preferably at least 110°C, is generally sufficient to ensure hollowness and the temperature may be as high as 250°C without the capsules bursting. About 180-240°C, preferably about 210-230°C and most preferably about 220°C, is optimal, at least for albumin. The temperature may, in the one step version of the process of the invention, be sufficient to insolubilise at least part (usually the outside) of the wall-forming material and frequently substantially all of the wall-forming material. Since the temperature of the gas encountered by the aerosol will depend also on the rate at which the aerosol is delivered and on the liquid content of the protein preparation, the outlet temperature may be

monitored to ensure an adequate temperature in the chamber. An outlet temperature of 40-150°C has been found to be suitable. Apart from this factor, however, controlling the flow rate has not been found to be as useful as controlling the other parameters.

In the two step process, the intermediate microcapsules comprise typically 96-98% monomeric HA and have a limited *in vivo* life time for ultrasound imaging. They may, however, be used for ultrasound imaging, or they may be stored and transported before the second step of the two step process is carried out. They therefore form a further aspect of the invention.

In the second step of the process, the intermediate microcapsules prepared in the first step are fixed and rendered less water-soluble so that they persist for longer whilst not being so insoluble and inert that they are not biodegradable. This step also strengthens the microcapsules so that they are better able to withstand the rigours of administration, vascular shear and ventricular pressure. If the microcapsules burst, they become less echogenic. Schneider *et al* (1992) *Invest. Radiol.* 27, 134-139 showed that prior art sonicated albumin microbubbles do not have this strength and rapidly lose their echogenicity when subjected to pressures typical of the left ventricle. The second step of the process may employ heat (for example microwave heat, radiant heat or hot air, for example in a conventional oven), ionising irradiation (with, for example, a 10.0-100.0 kGy dose of gamma rays) or chemical cross-linking using, for example, formaldehyde, glutaraldehyde, ethylene oxide or other agents for cross-linking proteins and is carried out on the substantially

dry intermediate microcapsules formed in the first step, or on a suspension of such microcapsules in a liquid in which the microcapsules are insoluble, for example a suitable solvent. In the one step version of the process, a cross-linking agent such as glutaraldehyde may be sprayed into the spray-drying chamber or may be introduced into the protein preparation just upstream of the spraying means. Alternatively, the temperature in the chamber may be high enough to insolubilise the microcapsules.

The final product, measured in the same way as the intermediate microcapsules, may, if one wishes, consist of microcapsules having a diameter of 0.05 to 50.0 μm, but ranges of 0.1 to 20.0 μm and especially 1.0 to 8.0 μm are obtainable with the process of the invention and are preferred for echocardiography. We have found that a range of about 0.5 to 3.0 μm may be especially suitable for the production of a low contrast image and for use in colour Doppler imaging, whereas a range of about 4.0 to 6.0 μm may be better for the production of sharp images. One needs to take into account the fact that the second step may alter the size of the microcapsules in determining the size produced in the first step.

It has been found that the process of the invention can be controlled in order to obtain microspheres with desired characteristics. Thus, the pressure at which the protein solution is supplied to the spray nozzle may be varied, for example from 1.0-10.0 x 10^5 Pa, preferably 2.0-6.0 x 10^5 Pa and most preferably about 5 x 10^5 Pa. Other parameters may be varied as disclosed above and below. In this way, novel microspheres may be obtained.

A further aspect of the invention provides hollow microspheres in which more than 30%, preferably more than 40%, 50%, or 60%, of the microspheres have a diameter within a 2 μm range and at least 90%, preferably at least

95% or 99%, have a diameter within the range 1.0-8.0 μm.

Thus, the interquartile range may be 2 μm, with a median diameter of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 μm.

Thus, at least 30%, 40%, 50% or 60% of the microspheres may have diameters within the range 1.5-3.5 μm, 2.0-4.0 μm, 3.0-5.0 μm, 4.0-6.0 μm, 5.0-7.0 μm or 6.0-8.0 μm. Preferably a said percentage of the microspheres have diameters within a 1.0 μm range, such as 1.5-2.5 μm, 2.0-3.0 μm, 3.0-4.0 μm, 4.0-5.0 μm, 5.0-6.0 μm, 6.0-7.0 μm or 7.0-8.0 μm.

A further aspect of the invention provides hollow microspheres with proteinaceous walls in which at least 90%, preferably at least 95% or 99%, of the microspheres have a diameter in the range 1.0-8.0 μm; at least 90%, preferably at least 95% or 99%, of the microspheres have a wall thickness of 40-500 nm, preferably 100-500 nm; and at least 50% of the protein in the walls of the microspheres is cross-linked. Preferably, at least 75%, 90%, 95%, 98.0%, 98.5% or 99% of the protein is sufficiently cross-linked to be resistant to extraction with a 1% HCl solution for 2 minutes. Extracted protein is detected using the Coomassie Blue protein assay, Bradford. The degree of cross-linking is controlled by varying the heating, irradiation or chemical treatment of the protein. During the cross-linking process, protein monomer is cross-linked and quickly becomes unavailable in a simple dissolution process, as detected by gel permeation HPLC or gel electrophoresis, as is shown in Example 8 below. Continued treatment leads to further cross-linking of already cross-linked material such that it becomes unavailable in the HCl extraction described above. During heating at 175°C, rHA microspheres in accordance with the invention lose about 99% of HCl-extractable protein over the course of 20 minutes, whereas, at 150°C, 20 minutes' heating removes

only about 5% HCl-extractable protein, 30 mins removes 47.5%, 40 mins 83%, 60 mins 93%, 80 mins 97% and 100 mins removes 97.8% of the HCl-extractable protein. To achieve good levels of cross-linking therefore, the microspheres may be heated at 175°C for at least 17-20 mins, at 150°C for at least 80 mins and at other temperatures for correspondingly longer or shorter times.

A further aspect of the invention provides hollow microspheres predominantly of 1.0-10.0 μm in diameter, at least 10% of the microspheres, when suspended in water, being capable of surviving a 0.25 s application of a pressure of 2.66×10^4 Pa without bursting, collapsing or filling with water. The transient maximum pressure in the human left ventricle is about 200 mmHg (2.66×10^4 Pa). Preferably 50%, 75%, 90% or 100% survive the said 0.25 s application of 2.66×10^4 Pa when tested as above, ie remain echogenic. *In vivo*, preferably the same percentages will remain echogenic during one passage through both ventricles of the heart.

The injectable microspheres of the present invention can be stored dry in the presence or in the absence of additives to improve conservation and prevent coalescence. As additives, one may select from 0.1 to 25% by weight of water-soluble physiologically acceptable compounds such as mannitol, galactose, lactose or sucrose or hydrophilic polymers like dextran, xanthan, agar, starch, PVP, polyglutamic acid, polyvinylalcohol (PVA) and gelatin. The useful life-time of the microspheres in the injectable liquid carrier phase, ie the period during which useful echographic signals are observed, can be controlled to last from a few minutes to several months depending on the needs; this can be done by controlling the porosity, solubility or degree of cross-linking of the wall. These parameters can be controlled by properly selecting the wall-forming materials and additives and by adjusting the

evaporation rate and temperature in the spray-drying chamber.

In order to minimise any agglomeration of the microcapsules, the microcapsules can be milled with a suitable inert excipient using a Fritsch centrifugal pin mill equipped with a 0.5 mm screen, or a Glen Creston air impact jet mill. Suitable excipients are finely milled powders which are inert and suitable for intravenous use, such as lactose, glucose, mannitol, sorbitol, galactose, maltose or sodium chloride. Once milled, the microcapsules/excipient mixture can be suspended in aqueous medium to facilitate removal of non-functional/defective microcapsules. Upon reconstitution in the aqueous phase, it is desirable to include a trace amount of surfactant to prevent agglomeration. Anionic, cationic and non-ionic surfactants suitable for this purpose include poloxamers, sorbitan esters, polysorbates and lecithin.

The microcapsule suspension may then be allowed to float, or may be centrifuged to sediment any defective particles which have surface defects which would, in use, cause them to fill with liquid and be no longer echogenic.

The microcapsule suspension may then be remixed to ensure even particle distribution, washed and reconstituted in a buffer suitable for intravenous injection such as 0.15M NaCl 0.01 mM Tris pH 7.0. The suspension may be aliquoted for freeze drying and subsequent sterilisation by, for example, gamma irradiation, dry heating or ethylene oxide.

An alternative method for deagglomeration of the insolubilised or fixed microcapsules is to suspend them directly in an aqueous medium containing a surfactant chosen from poloxamers, sorbitan esters, polysorbates and lecithin. Deagglomeration may then be achieved using a suitable homogeniser.

The microcapsule suspension may then be allowed to float or may be centrifuged to sediment the defective particles, as above, and further treated as above.

Although the microspheres of this invention can be marketed in the dry state, more particularly when they are designed with a limited life time after injection, it may be desirable to also sell ready-made preparations, ie suspensions of microspheres in an aqueous liquid carrier ready for injection.

The product is generally, however, supplied and stored as a dry powder and is suspended in a suitable sterile, non-pyrogenic liquid just before administration. The suspension is generally administered by injection of about 1.0-10.0 ml into a suitable vein such as the cubital vein or other bloodvessel. A microcapsule concentration of about 1.0×10^5 to 1.0×10^{12} particles/ml is suitable, preferably about 5.0×10^5 to 5.0×10^9.

Although ultrasonic imaging is applicable to various animal and human body organ systems, one of its main applications is in obtaining images of myocardial tissue and perfusion or blood flow patterns.

The techniques use ultrasonic scanning equipment consisting of a scanner and imaging apparatus. The equipment produces visual images of a predetermined area, in this case the

heart region of a human body. Typically, the transducer is placed directly on the skin over the area to be imaged. The scanner houses various electronic components including ultrasonic transducers. The transducer produces ultrasonic waves which perform a sector scan of the heart region. The ultrasonic waves are reflected by the various portions of the heart region and are received by the receiving transducer and processed in accordance with pulse-echo methods known in the art. After processing, signals are sent to the imaging apparatus (also well known in the art) for viewing.

In the method of the present invention, after the patient is "prepped" and the scanner is in place, the microcapsule suspension is injected, for example through an arm vein. The contrast agent flows through the vein to the right venous side of the heart, through the main pulmonary artery leading to the lungs, across the lungs, through the capillaries, into the pulmonary vein and finally into the left atrium and the left ventricular cavity of the heart.

With the microcapsules of this invention, observations and diagnoses can be made with respect to the amount of time required for the blood to pass through the lungs, blood flow patterns, the size of the left atrium, the competence of the mitral valve (which separates the left atrium and left ventricle), chamber dimensions in the left ventricular cavity and wall motion abnormalities. Upon ejection of the

contrast agent from the left ventricle, the competence of the aortic valve also may be analyzed, as well as the ejection fraction or percentage of volume ejected from the left ventricle. Finally, the contrast patterns in the tissue will indicate which areas, if any, are not being adequately perfused.

In summary, such a pattern of images will help diagnose unusual blood flow characteristics within the heart, valvular competence, chamber sizes and wall motion, and will provide a potential indicator of myocardial perfusion.

The microcapsules may permit left heart imaging from intravenous injections. The albumin microcapsules, when injected into a peripheral vein, may be capable of transpulmonary passage. This results in echocardiographic opacification of the left ventricle (LV) cavity as well as myocardial tissue.

Besides the scanner briefly described above, there exist other ultrasonic scanners, examples of which are disclosed in US Patents Nos. 4,134,554 and 4,315,435, the disclosures of which are herein incorporated by reference. Basically, these patents relate to various techniques including dynamic cross-sectional echography (DCE) for producing sequential two-dimensional images of cross-sectional slices of animal or human anatomy by means of ultrasound energy at a frame rate sufficient to enable dynamic visualisation of

moving organs. Types of apparatus utilised in DCE are generally called DCE scanners and transmit and receive short, sonic pulses in the form of narrow beams or lines. The reflected signals' strength is a function of time, which is converted to a position using a nominal sound speed, and is displayed on a cathode ray tube or other suitable devices in a manner somewhat analogous to radar or sonar displays. While DCE can be used to produce images of many organ systems including the liver, gall bladder, pancreas and kidney, it is frequently used for visualisation of tissue and major blood vessels of the heart.

The microcapsules may be used for imaging a wide variety of areas, even when injected at a peripheral venous site. Those areas include (without limitation): (1) the venous drainage system to the heart; (2) the myocardial tissue and perfusion characteristics during an exercise treadmill test or the like; and (3) myocardial tissue after an oral ingestion or intravenous injection of drugs designed to increase blood flow to the tissue. Additionally, the microcapsules may be useful in delineating changes in the myocardial tissue perfusion due to interventions such as (1) coronary artery vein grafting; (2) coronary artery angioplasty (balloon dilation of a narrowed artery); (3) use of thrombolytic agents (such as streptokinase) to dissolve clots in coronary arteries; or (4) perfusion defects or changes due to a recent heart attack.

Furthermore, at the time of a coronary angiogram (or a digital subtraction angiogram) an injection of the microcapsules may provide data with respect to tissue perfusion characteristics that would augment and complement the data obtained from the angiogram procedure, which identifies only the anatomy of the blood vessels.

Through the use of the microcapsules of the present invention, other non-cardiac organ systems including the liver, spleen and kidney that are presently imaged by ultrasonic techniques may be suitable for enhancement of such currently obtainable images, and/or the generation of new images showing perfusion and flow characteristics that had not previously been susceptible to imaging using prior art ultrasonic imaging techniques.

Preferred aspects of the present invention will now be described by way of example and with reference to

Figure 1, which is a partly cut away perspective view from the front and one side of suitable spray-drying apparatus for the first stage of the process of the invention,

Figure 2, which is a graph showing how the degree of fixation of the microsphere walls (in this case albumin) may be controlled by varying the temperature and the heating time in the second step of the process, and

Figure 3, which is a graph showing how the pressure resistivity of the microspheres may be varied by altering the length of the heating time in the second step of the process.

EXAMPLE 1

A suitable spray dryer (Figure 1) is available from A/S Niro Atomizer, Soeborg, Denmark under the trade designation "Mobile Minor". Details of its construction are given immediately before the claims herein. It comprises a centrifugal atomizer (Type M-02/B Minor), driven by an air turbine at an air pressure of min 4 bar and up to max 6 bar. At 6 bar an atomizer wheel speed of approx 33,000 rpm is reached. Turning on and off the compressed air to the atomizer is done by means of a valve placed in the instrument panel. The maximum consumption of compressed air to the atomizer is 17 Nm^3/h at a pressure of 6 bar. All parts coming into contact with the liquid feed and powder are made of stainless steel AISI 316, except for the pump feed tube and the atomizer wheel, which is made of stainless steel AISI 329, made to resist high centrifugal force.

The drying chamber has an inside made of stainless steel AISI 316, well insulated with Rockwool, and covered outside with a mild steel sheeting. The drying chamber is provided with a side light and observation pane for inspection during the operation. The roof of the drying chamber is made inside of stainless steel AISI 316 and outside of stainless steel AISI 304.

An air disperser made of stainless steel AISI 304 is used for distribution of the air in the drying chamber in order to achieve the best possible drying effect. An air duct, made of stainless steel AISI 316, provides lateral transportation of the exhaust air and the powder to the cyclone, which is made of stainless steel AISI 316 and designed to separate the powder and air.

A closing valve of the butterfly valve type, also made of stainless steel AISI 316 and having a gasket of silicone rubber, is used for powder discharge under the cyclone into a powder collecting glass jar tightly placed under the cyclone by means of a spring device.

A fan made of silumin, complete with 3-phase squirrel-cage motor, 0.25 kW, and V-belt drive with belt-guard, draws air and powder through the drying chamber and cyclone.

An air heater heats the drying air by means of electricity (total consumption 7.5 kWh/h, infinitely variable) and can give inlet air temperatures of up to about 350°C, although this is generally too high for preparing the microcapsules of the invention.

Equipment for two-fluid nozzle atomization may be added, which is made of stainless steel AISI 316, consisting of entrance pipe with nozzle holder and nozzle, to be placed in the ceiling of the drying chamber. The equipment

includes an oil/water separator, reduction valve and pressure gauge for compressed air to the two-fluid nozzle. Consumption of compressed air: 8-15 kg/h at a pressure of 0.5-2.0 bar (0.5-2.0 x 10^5 Pa).

A suitable feed pump for transport of wall-forming preparation feed to the atomizer device is a peristaltic pump. The pump is provided with a motor (1 x 220V, 50 Hz, 0.18 kW) and a continuously variable gear for manual adjustment. A feed pipe made of silicone hose leads from a feed tank (local supply) through the feed pump to the atomization device.

An absolute air filter, consisting of prefilter, filter body in stainless steel and absolute air filter, is used for the treatment of the ingoing drying air to render it completely clean.

A 20% solution of sterile, pyrogen-free rHA in pyrogen-free water (suitable for injection) was pumped to the nozzle of a two fluid nozzle atomiser mounted in the commercial spray drying unit described above. The peristaltic pump speed was maintained at a rate of approximately 10 ml/minute such that with an inlet air temperature of 220°C the outlet air temperature was maintained at 95°C.

Compressed air was supplied to the two fluid atomising nozzle at 2.0-6.0 Bar (2.0-6.0 x 10^5Pa). In this range

microcapsules with a mean size of 4.25-6.2 μm are obtained.

Typically an increase in mean particle size (by reduced atomisation pressure) led to an increase in the amount of microcapsules over 10 μm in size (see Table 1).

TABLE 1

EFFECTS OF ATOMISATION PRESSURE ON FREQUENCY OF MICROCAPSULES OVER 10 μM IN DIAMETER

Atomisation Pressure (x 10^5 Pa)	% Frequency over 10 μm
6.0	0.8
5.0	0.3
3.5	6.6
2.5	8.6
2.0	13.1

In the second step of the process, 5 g of microcapsules were heated in a glass beaker using a Gallenkamp fan oven. A temperature of 175°C for 1 hour was sufficient to yield microcapsules with 100% fixation as determined by HPLC. The effect of this heat fixation was to increase the *in vitro* echogenic half life from a few seconds to in excess of 30 minutes. By altering the temperature and length of

incubation it is possible to vary the degree of fixation between about 5% and 100%. Examples of heat fixation profiles of varying temperatures are shown in Figure 2.

Following heat fixation, the microcapsules were deagglomerated and dispersed into water in one of two ways. Method 1 involved first mixing the heat fixed spheres with an equal weight of finely milled lactose (mean diameter 5 μm). The mixture was then passed through a Fritsch centrifugal mill with a 0.5 mm screen and 12 tooth rotor. The milled spheres were collected and passed through the mill a second time to ensure complete mixing had occurred. The milled powder was then resuspended in water containing 1 $mg.ml^{-1}$ Pluronic F68. Typically 10 g of microcapsules and lactose was added to 100 ml of water and Pluronic F68. Method 2 for deagglomeration involves adding 5 g of the heat-fixed microcapsules to 100 ml of water containing 100 mg of Pluronic F68. The microcapsules were dispersed using a Silverson homogeniser (model L4R with a 2.54 cm tubular homogenising probe and a high shear screen) and homogenising for 60 seconds.

The resuspended spheres were separated into intact (gas containing) and broken spheres using a flotation technique. The gas-containing spheres were seen to float to the surface over a 1 hour period and were decanted from the sinking fraction which does not contain the gas required.

The separation process can be accelerated by centrifugation. A 30 second centrifugation at 5000 x g is sufficient to separate the two fractions.

Following separation the intact microcapsules were freeze-dried in the presence of lactose and Pluronic F68. Optimal conditions for freeze drying involved resuspending 30 mg of microcapsules in 5 ml of water containing 50 mg of lactose and 5 mg of Pluronic F68. The freeze-dried microcapsules can be redispersed in a liquid (eg water, saline) to give a monodisperse distribution.

EXAMPLE 2

The process of Example 1 was repeated but with the following differences in the first step: a centrifugal atomiser was used instead of a two fluid nozzle; the inlet temperature was 150°C (with the outlet air temperature still being sustained at 105°C); and compressed air was supplied to the nozzle at 1.0-6.0 x 10^5 Pa. The wheel rotated at 20-40,000 rpm and delivered droplets, and subsequently microcapsules, with a number mean diameter in the 1.0-8.0 μm range.

EXAMPLE 3

In the second step of the process of Example 1 or 2 was varied as follows. A small aliquot of the microcapsules

(0.5 g) was heated in a microwave oven such that it received 300-350 watt hours of microwave heat at 2500 mHz. This yielded microcapsules in which 90-95% of the monomeric rHA was insoluble (as determined by gel permeation chlomatography) and as a result of this heat fixation their *in vitro* echogenic half-life increased from a few seconds to in excess of 30 minutes.

EXAMPLE 4

In the seoncd step of the process of Example 1 or 2 was varied as follows. A small aliquot of the microcapsules (0.5 g) was sealed under argon in a glass vial. The vial was cooled to 4°C and then irradiated with a ^{60}Co gamma radiation source to deliver a 15.0 kGy dose of gamma rays. The irradiation resulted in the formation of microcapsules in which 10-15% of the monomeric albumin was insoluble.

EXAMPLE 5

In the second step of the process of Example 1 or 2 was varied as follows. A small aliquot of the microcapsules (0.5 g) was sealed under argon in a glass vial. The vial was cooled to 4°C and then irradiated with a ^{60}Co gamma radiation source to deliver a 50.0 kGy dose of gamma rays to the microcapsules. Following irradiation, the microcapsules were incubated in oxygen at 50°C for 6 hours. The irradiation resulted in the formation of microcapsules

in which 50-60% of the monomeric rHA was insoluble.

EXAMPLE 6

In the second step of the process of Example 1 or 2 was varied as follows.

A small aliquot of microcapsules (0.5 g) was resuspended in 5 ml of ethanol, chloroform or methylene chloride containing a) 1.5% glutaraldehyde, b) 2.0% diphthaloyl chloride or c) 5.0% formaldehyde. The microcapsules were stirred for varying times from 10 minutes to 3 hours. The microcapsules were removed by filtration and washed thoroughly in the original organic buffer containing 5% ethanolamine, in order to remove excess cross-linking agent. Finally the microcapsules were washed in organic solvent and vacuum dried to remove any residual solvents. The extent of insolubilisation may be varied from 5-100% by this method resulting in the extension of *in vitro* echogenic half-life from 1-2 minutes to in excess of one hour.

EXAMPLE 7

The two independent steps of microcapsule formation and insolubilisation of the shell may be combined in a single process. In this example, the formation of the microcapsules and the insolubilisation of the polymeric

material are achieved simultaneously during the spray drying process.

A solution of rHA was fed by peristaltic pump to a small reaction chamber, with a separate feed line supplying a 5% solution of a suitable crosslinking agent, eg glutaraldehyde, diphthaloyl chloride or formaldehyde. The residence time in the reaction chamber was such that initial adduct formation between the crosslinking agent and the protein was achieved, but intraprotein crosslinking was prevented. The reaction vessel outlet was fed directly to the two fluid nozzle atomisers mounted in a specially adapted spray drying unit, capable of handling volatile solvents. The conditions of spray drying were as outlined in Example 1. The microcapsules were incubated dry at room temperature to allow intraprotein crosslinks to form and then suspended in ethanol containing 5% ethanolamine to quench any remaining crosslinking agent. Thorough washing of the microcapsules was performed and finally the microcapsules were vacuum dried to remove residual solvent.

EXAMPLE 8: ASSAY OF FREE MONOMERIC rHA IN MICROCAPSULES

A 1 ml volume of ethanol was added to 100 mg of microcapsules in a 20 ml glass bottle and sonicated for 30 seconds. To this suspension 19 ml of H_2O were added.

The mixture was centrifuged in a bench-top microfuge

(Gilson) for 20 seconds and the clear fraction assayed. The assay was performed by loading 50 ml of the fraction automatically onto a Shimadzu LC6A HPLC and chromatographing on a TSK gel permeation column at a flow rate of 1 ml minute^{-1} using sodium phosphate buffer (pH 7.0).

The peak heights representing the rHA monomer were recorded and used to determine the concentration of monomer using a standard curve between 1 and 10 mgml^{-1} monomeric rHA.

The %-free monomeric rHA was calculated by measuring the monomer concentration in the fixed microcapsules and representing this figure as a percentage of the monomer concentration of the unfixed microcapsules. The results are given in Figure 2.

Heating of the spray dried microcapsules in an oven (as described in Example 1) results in a decrease in the amount of monomer that can be detected (see Figure 2). This decrease in detectable monomeric rHA is due to the denaturation and crosslinking of monomeric rHA into insoluble polymers that cannot be assayed by the aforementioned HPLC method.

Using the HPLC method to assess rHA monitor levels, it is clear from Figure 2 that after 15 minutes incubation there is no free monomeric rHA present in the rHA microcapsules. However it is still possible to further crosslink the rHA

microcapsules by heating for longer periods.

This prolonged heating results in an increased level of microcapsule crosslinking which in turn produces microcapsules of increasing strength which are correspondingly more resistant to pressure.

By careful control of temperature and time of incubation, it is possible to produce microcapsules with a controlled range of crosslinking (and hence pressure resistivity).

EXAMPLE 9: EFFECTS OF INCUBATION TIME AT 175°C ON THE PRESSURE RESISTIVITY OF rHA MICROCAPSULES

A batch of rHA microcapsules was divided into 5 g aliquots and baked at 175°C for varying lengths of time as shown in Figure 3.

Following heat fixation the amount of free monomer was determined as described in Example 8. For each of the incubations shown, there was no monomeric rHA detectable.

The heat-fixed microcapsules were disaggregated using a Fritsch centrifugal mill (as described above) and intact, air-containing microcapsules recovered by the aforementioned flotation technique. The recovered microcapsules were suspended in H_2O containing Pluronic F68 (1 $mgml^{-1}$) at a concentration of 0.5 x 10^8 capsules ml^{-1}.

The resuspended, air-containing microcapsules were subjected to increased atmospheric pressure by applying pressure with a 50 ml syringe whilst containing this suspension in a closed container (25 ml polystyrene container).

For each of the pressures assessed, the individual microcapsule suspension was pressurised to the selected pressure and maintained at this pressure for 5 seconds before releasing the pressure. For each suspension analysed the pressure increase was performed 3 times. The pressure in the closed container was assessed by an RS hand-held manometer.

Following pressurisation the microcapsule suspensions were assessed by light microscopy and image analysis and the % air-containing to non-air-containing microcapsules assessed. This analysis is performed since only the air-containing microcapsules are functional in enhancing ultrasound echocontrast.

As can be seen in Figure 3, microcapsules that are fixed for 60 minutes at 175°C, as described in Example 1, are stable at all of the pressures to which they were subjected in this experiment.

By careful control of the length of incubation at this particular temperature (175°C) it is possible to produce

batches of microcapsules with different degrees of crosslinking which in turn are resistant to varying degrees of pressure increase.

Using this careful control of crosslinking by adjusting the length and temperature of incubation it is possible to produce batches of air-containing microcapsules that are specifically designed to withstand a designated pressure increase.

The temperature used to crosslink the microcapsules can vary infinitely, as can the length of incubation time.

EXAMPLE 10: MICROCAPSULE CLASSIFICATION

An advantage of the process of the invention is that it enables the median size and size distribution of the microspheres to be controlled. However, one can further select desired sizes if one wishes, for example by flotation. In a homogeneous dispersion of microspheres, larger particles will rise to the surface faster than smaller particles due to the lower density (more encapsulated air) of the larger particles. Hence, by allowing the dispersion to stand, the particle size distribution will change at any level of the solution with respect to time.

Microcapsules were dispersed in 2000 ml of aqueous solution

containing 6% w/v sodium chloride and 0.1% w/v Pluronic F68 in a glass bottle giving a liquid column of approximately 165 mm. A sampling tube was placed 50 mm below the upper liquid surface to enable removal of samples at timed intervals.

By altering the standing time and sodium chloride concentration, it was possible to produce a variety of particle size distributions and classify microcapsules down to 2 μm.

Other wet techniques for classification include hydrodynamic chromatography and field flow fractionation. 'Dry' techniques using the principles of elutriation and cross flow separation are commercially available in the form of the Microsplit (British Rem.), Zig-zag (Alpine) and Turbo (Nissuin) classifiers. The elbow jet classifier produced by Nitettsu Mining Co uses a different principle (the Coanda Effect) which could also achieve good results for the classification of microcapsules.

Further details of construction of atomiser

In Figure 1, reference numeral 1 denotes the feeding device. 2 is a ceiling air disperser which ensures effective control of the air flow pattern. Swirling air is directed around the vaned disc atomiser. 3 is a rotary atomiser or nozzle atomiser. 4 shows a stainless steel

interconnecting pipe system which can easily be stripped down for cleaning. 5 are steps for access to the chamber top. 6 is the switch for an air valve for activation of the pneumatic lifting device when raising the chamber lid. 7 is a highly-efficient stainless steel cyclone in which the powder and the exhausted drying air are separated. 8 is a glass jar in which the powder is recovered. 9 is a centrally located instrument panel. 10 is a centrifugal exhaust fan with 3-phase motor. 11 is a damper for air flow control and 12 is an electric air heater which provides drying air temperatures up to 350°C. The drying air temperature can be continuously adjusted using a percentage timer switch. The maximum powder consumption is 7.5 kW.

Evaporative capacity

Drying Air	Inlet Air Temperature	Outlet Air Temperature	Evaporative Capacity
85 kg/h	150°C	80°C	1,3 kg/h
85 kg/h	170°C	85°C	1,7 kg/h
80 kg/h	200°C	90°C	2,5 kg/h
80 kg/h	240°C	90°C	3,4 kg/h
75 kg/h	350°C	90°C	7,0 kg/h

Weight and dimension

Weight	280 kgs
Length	1800 mm
Height	2200 mm
Width	925 mm

Power. The unit can only be operated on a 3-phase power supply (50 or 60 Hz) at alternative voltages of 440, 415, 400, 380, 220, 200 V.

All parts coming into contact with the liquid or the product are made of acid-resistant, stainless steel AISI 316.

CLAIMS

1. A process comprising the step of atomising a solution or dispersion of a wall-forming material in a liquid carrier into a gas in order to obtain hollow microcapsules by evaporation of the liquid carrier.

2. A process according to Claim 1 wherein the product obtained thereby is subjected to the further step of reducing the water-solubility of at least the outside of the microcapsules.

3. A process according to Claim 1 or 2 wherein the wall-forming material is a protein.

4. A process according to Claim 3 wherein the protein is collagen, gelatin or serum albumin.

5. A process according to Claim 4 wherein the protein is human serum albumin, or an analogue or fragment thereof, prepared by recombinant DNA techniques.

6. A process according to any one of Claims 3 to 6 wherein the protein solution or dispersion comprises 10.0-30.0% protein.

7. A process according to any one of Claims 3 to 6 wherein in the step of Claim 1 the protein solution or

dispersion is atomised as said to form discrete microcapsules of 0.01-50.0 μm diameter.

8. A process according to any one of Claims 3 to 7 wherein the product of the process of Claim 1 comprises 96-98% monomeric protein.

9. A process according to any one of Claims 3 to 8 when dependent on Claim 2 wherein the product of the step of Claim 2 comprises no more than 5% monomeric protein.

10. A process according to any one of Claims 2 to 7 wherein the conditions of the step of Claim 1 are such as to achieve the step of Claim 2 substantially simultaneously.

11. Microcapsules obtained by a process according to any one of Claims 1 to 10.

12. Microcapsules obtainable by a process according to any one of Claims 1 to 10.

13. Hollow microspheres in which more than 30% of the microspheres have a diameter within a 2 μm range and at least 90% have a diameter within the range 1.0-8.0 μm.

14. Hollow microspheres in which the interquartile range of diameters is 2 μm or less and the median diameter is

between 2.0 μm and 8.0 μm inclusive.

15. Hollow microspheres with proteinaceous walls in which at least 90% of the microspheres have a diameter in the range 1.0-8.0 μm; at least 90% of the microspheres have a wall thickness of 40-500 nm; and at least 50% of the protein in the walls of the microspheres is so cross-linked as to be resistant to extraction in 1% HCl for 2 mins.

16. Hollow microspheres according to Claim 15 wherein at least 95% of the protein is cross-linked as said.

17. Hollow microspheres predominantly of 1.0-10.0 μm in diameter, at least 10% of the microspheres, when suspended in water, being capable of surviving a 0.25 s application of a pressure of 2.66×10^4 Pa without bursting, collapsing or filling with water.

18. A method of generating an image for subsequent inspection, comprising (a) injecting into the body of a mammal microcapsules according to any one of Claims 11 to 17, (b) subjecting the mammal or part thereof to suitable ultrasonic radiation and (c) detecting ultrasonic radiation reflected, transmitted, resonated or frequency modulated by the said microcapsules.

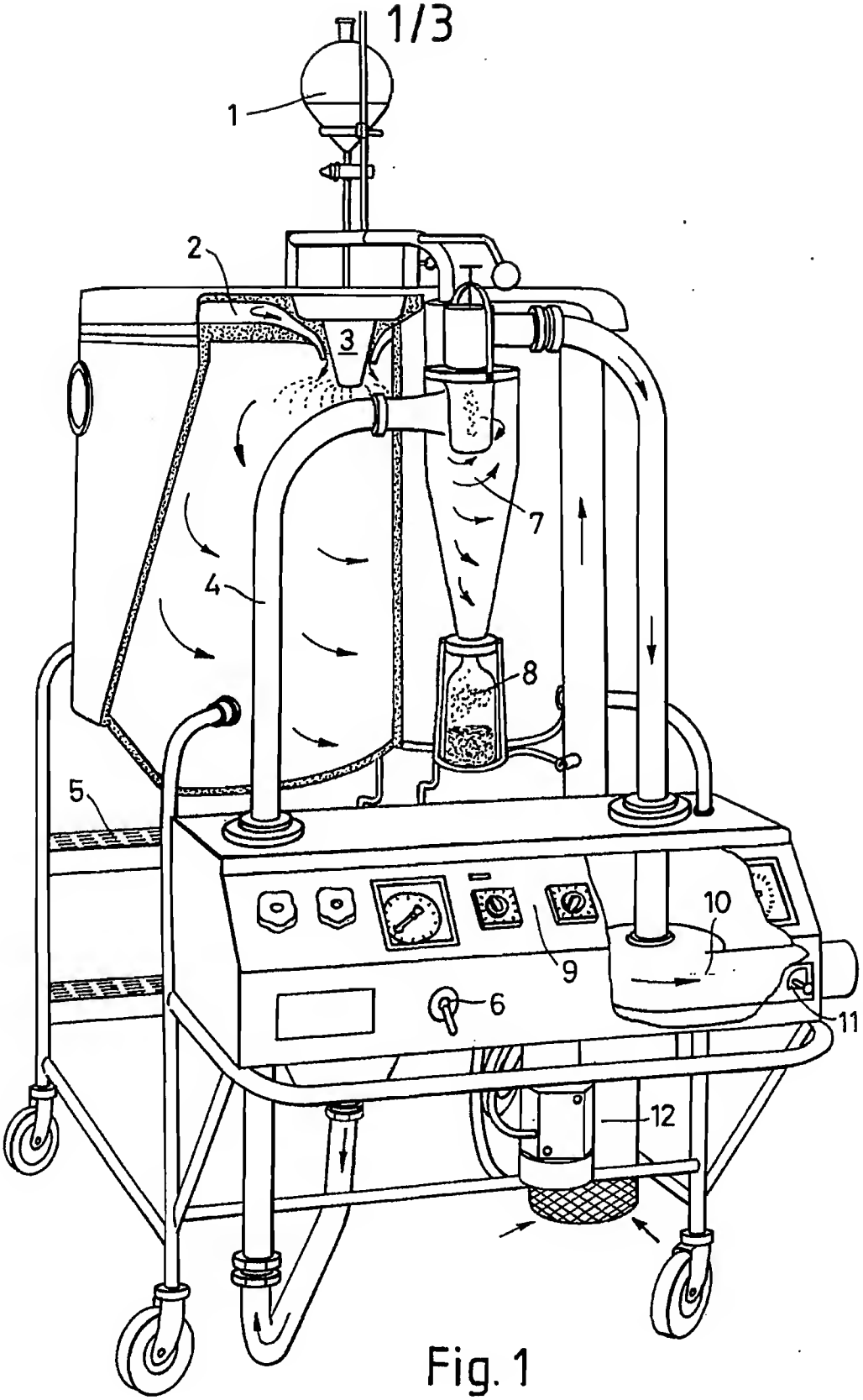
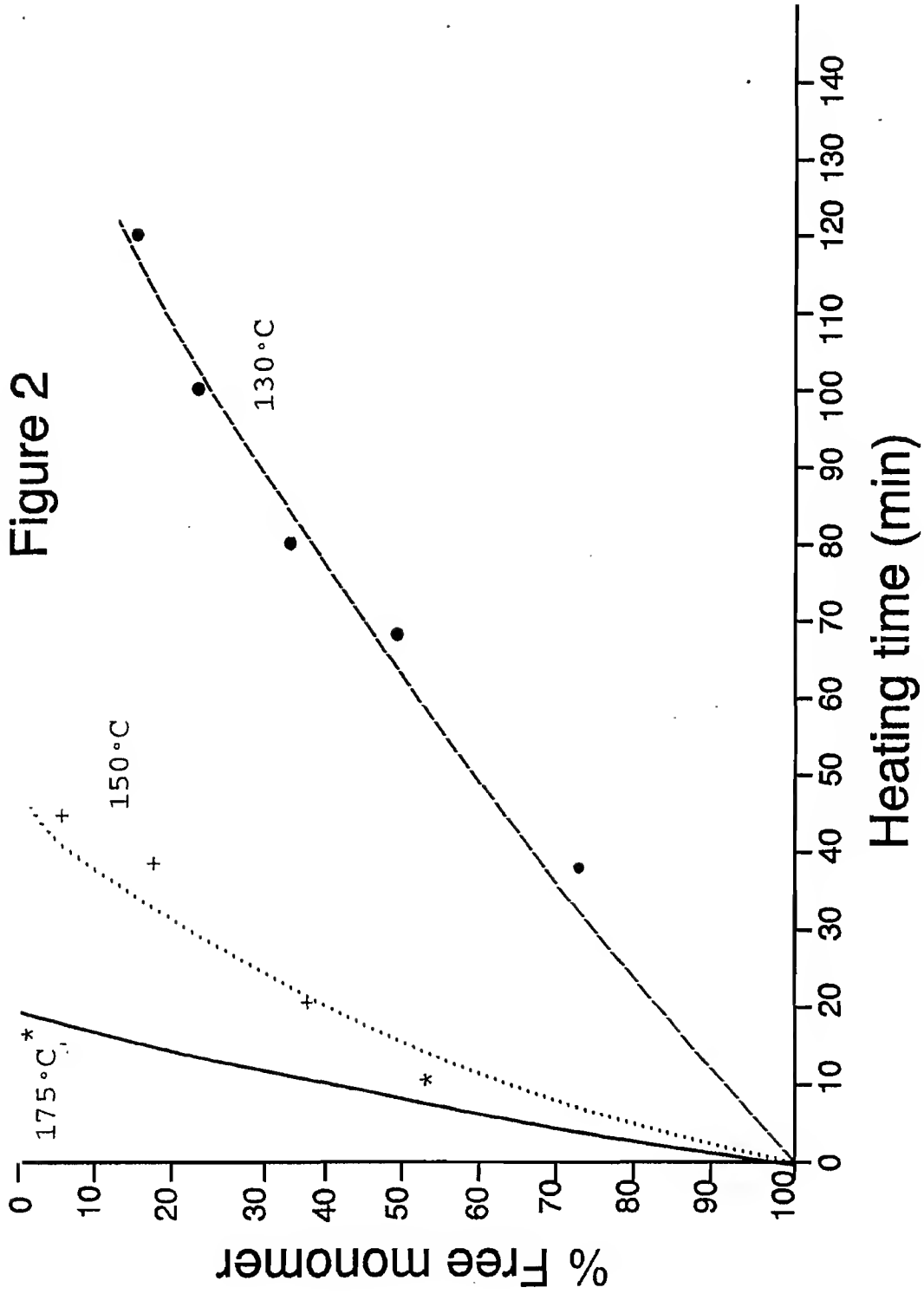


Fig. 1

Figure 2


% Free monomer
Heating time (min)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
175°C
150°C
130°C

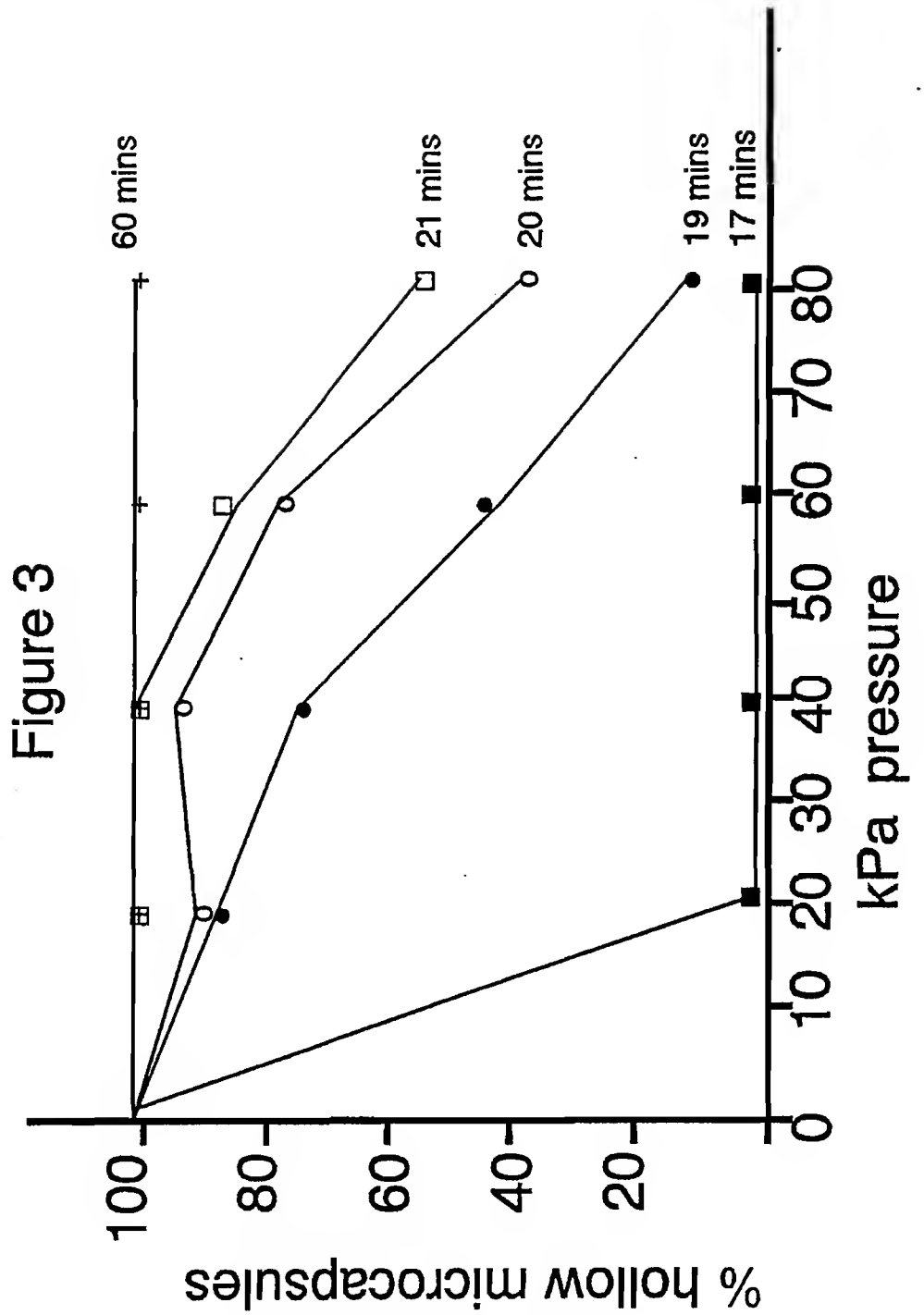

% hollow microcapsules
100
80
60
40
20
0
10
20
30
40
50
60
70
80
kPa pressure
60 mins
21 mins
20 mins
19 mins
17 mins

Figure 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00643

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 49/00 B 01 J 13/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K . B 01 J

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X,Y	US,A,4089800 (R.G. TEMPLE) 16 May 1978, see column 2, lines 5-25; claims; column 9, lines 56-66, column 1, lines 5-31 ---	1-17
A,P	WO,A,9112823 (DELTA BIOTECHNOLOGY) 5 September 1991, see page 5, column 2; claims 1,6,15,16 (cited in the application) ---	1-17
A	Journal of Controlled Release, vol. 16, 1991, Elsevier Science Publishers B.V., Y. KAWASHIMA et al.: "Preparation of multiple unit hollow microspheres (microballoons) with acrylic resin containing tranilast and their drug release characteristics (in vitro) and floating behavior (in vivo)", pages 279-289, see page 286, figure 5 --- -/-	13-17

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02-06-1992	30. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0381543 (BIOETICA S.A.) 8 August 1990, see page 3, line 55 - page 6, line 10	1-17
Y	EP,A,0091555 (PQ CORP.) 19 October 1983, see page 7, lines 14-34; claims 17-18	1-17
X		13-17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 18 because they relate to subject matter not required to be searched by this Authority, namely:

Please see PCT-rule 39.1(iv):

Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200643 SA 58288

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/06/92
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A- 4089800	16-05-78	None		
WO-A- 9112823	05-09-91	None		
EP-A- 0381543	08-08-90	FR-A-	2642329	03-08-90
		AU-A-	4886490	09-08-90
		JP-A-	2229111	11-09-90
EP-A- 0091555	19-10-83	CA-A-	1221586	12-05-87
		JP-A-	58177133	17-10-83
		US-A-	4540629	10-09-85

EXHIBIT B

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) **International Patent Classification** 6 : **A61K 9/16, 9/50, 9/00** — **A1**

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94307126.6 29 September 1994 (29.09.94) EP
(34) Countries for which the regional or international application was filed: AT et al.

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(74) **Agent:** GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).

(81) **Designated States:** AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) **Title:** SPRAY-DRIED MICROPARTICLES AS THERAPEUTIC VEHICLES

(57) **Abstract**

Microparticles of a water-soluble material, which are smooth and spherical, and at least 90 % of which have a mass median particle size of 1 to 10 μm, and which either carry a therapeutic or diagnostic agent or use such an agent as the water-soluble material, can successfully be used in dry powder inhalers to deliver the said agent.

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SPRAY-DRIED MICROPARTICLES AS THERAPEUTIC VEHICLES

Field of the Invention

This invention relates to spray-dried microparticles and their use as therapeutic vehicles. In particular, the invention relates to means for delivery of diagnostic and therapeutic agents and biotechnology products, including therapeutics based upon rDNA technology.

Background of the Invention

The most commonly used route of administration of therapeutic agents, oral or gastrointestinal, is largely inapplicable to peptides and proteins derived from the rDNA industry. The susceptibility of normally blood-borne peptides and proteins to the acidic/proteolytic environment of the gut, largely precludes this route for administration. The logical means of administration is intravenous, but this presents problems of poor patient compliance during chronic administration and very often rapid first-pass clearance by the liver, resulting in short iv lifetimes.

Recently, the potential for delivery by mucosal transfer has been explored. Whilst nasal delivery has been extensively explored, the potential delivery of peptides via the pulmonary airways is largely unexplored.

Alveolar cells, in their own right, provide an effective barrier. However, even passage of material to the alveolar region represents a significant impediment to this method of administration. There is an optimal size of particle which will access the lowest regions of the pulmonary airways, i.e. an aerodynamic diameter of <5 μm. Particles above this size will be caught by impaction in the upper airways, such that in standard commercial suspension preparations, only 10-30% of particles, from what are normally polydispersed suspensions, reach the lowest airways.

Current methods of aerosolising drugs for inhalation include nebulisation, metered dose inhalers and dry powder systems. Nebulisation of aqueous solutions requires large

volumes of drugs and involves the use of bulky and non-portable devices.

The most common method of administration to the lung is by the use of volatile propellant-based devices, commonly termed metered dose inhalers. The basic design is a solution of propellant, commonly CFC 11, 12 or 114, containing either dissolved drug or a suspension of the drug in a pressurised canister. Dosing is achieved by depressing an actuator which releases a propellant aerosol of drug suspension or solution which is carried on the airways. During its passage to the lung, the propellant evaporates to yield microscopic precipitates from solution or free particles from suspension. The dosing is fairly reproducible and cheap, but there is growing environmental pressure to reduce the use of CFCs. Furthermore, the use of CFC solvents remains largely incompatible with many of the modern biotechnology drugs, because of their susceptibility to denaturation and low stability.

Concurrently, there is a move toward dry powder devices which consist of dry powders of drugs usually admixed with an excipient, such as lactose or glucose, which facilitates the aerosolisation and dispersion of the drug particles. The energy for disaggregation is often supplied by the breath or inspiration of air through the device.

Drugs are currently micronised, to reduce particle size. This approach is not applicable for biotechnology products. In general, biotechnology products are available in low quantity and, furthermore, are susceptible to the methods currently employed to dry and micronise prior to mixing with excipient. Further, it is particularly difficult to provide blends of drug and excipient which are sufficiently free-flowing that they flow and dose reproducibly in the modern multiple dose inhalers such as the Turbohaler (Astra) and Diskhaler (Glaxo). Studies have revealed that, contrary to expectation, spray-dried (spherical) salbutamol microparticles showed greater forces

of cohesion and adhesion than similarly-sized particles of micronised drug. Electron micrographs of the spray-dried material revealed the particles to possess pitted, rough surfaces.

Haghpanah et al reported, at the 1994 British Pharmaceutical Conference, that albumin microparticles incorporating salbutamol, had been produced by spray-drying and were of a suitable size for respiratory drug delivery, i.e. 1-5 μm. The aim was to encapsulate salbutamol, for slow release. It does not appear that the product is of substantially uniformly spherical or smooth microparticles that have satisfactory flow properties, for multi-dose dry powder inhalers.

Diagnostic agents comprising hollow microcapsules have been used to enhance ultrasound imaging. For example, EP-A-458745 (Sintetica) discloses a process of preparing air- or gas-filled microballoons by interfacial polymerisation of synthetic polymers such as polylactides and polyglycolides. WO-A-9112823 (Delta) discloses a similar process using albumin. Wheatley et al (1990) Biomaterials 11:713-717, disclose ionotropic gelation of alginate to form microbubbles of over 30 μm diameter. WO-A-9109629 discloses liposomes for use as ultrasound contrast agents.

Przyborowski et al, Eur. J. Nucl. Med. 7:71-72 (1982), disclose the preparation of human serum albumin (HSA) microspheres by spray-drying, for radiolabelling, and their subsequent use in scintigraphic imaging of the lung. The microspheres were not said to be hollow and, in our repetition of the work, predominantly poorly formed solid microspheres are produced. Unless the particles are hollow, they are unsuitable for echocardiography. Furthermore, the microspheres were prepared in a one-step process which we have found to be unsuitable for preparing microcapsules suitable for echocardiography; it was necessary in the prior process to remove undenatured albumin from the microspheres, and a wide size range of

microspheres was apparently obtained, as a further sieving step was necessary.

Przyborowski et al refer to two earlier disclosures of methods of obtaining albumin particles for lung scintigraphy. Aldrich & Johnston (1974) Int. J. Appl. Rad. Isot. 25:15-18, disclose the use of a spinning disc to generate 3-70 μm diameter particles which are then denatured in hot oil. The oil is removed and the particles labelled with radioisotopes. Raju et al (1978) Isotopenpraxis 14(2):57-61, used the same spinning disc technique but denatured the albumin by simply heating the particles. In neither case were hollow microcapsules mentioned, and the particles prepared were not suitable for echocardiography.

EP-A-0606486 (Teijin) describes the production of powders in which an active agent is incorporated into small particles, with carriers comprised of cellulose or cellulose derivatives. The intention is to prevent drug particles from adhering to the gelatin capsules used in a unit dose dry powder inhaler. Page 12 of this publication refers to the spray-drying of "medicament and base", to obtain particles of which 80% or more were 0.5-10 μm in size. No directions are given as to what conditions should be used, in order to obtain such a product.

EP-A-0611567 (Teijin) is more specifically concerned with the production of powders for inhalation, by spray-drying. The carrier is a cellulose, chosen for its resistance to humidity. The conditions that are given in Example 1 (ethanol as solvent, 2-5% w/v solute) mean that there is no control of surface morphology, and Example 4 reports a poor lower airway respirable fraction (12%), indicating poor dispersion properties. Spherical particles are apparently obtained at high drug content, indicating that particle morphology is governed by the respective drug and carrier contents.

Conte et al (1994) Eur. J. Pharm. Biopharm. 40(4):203-208, disclose spray-drying from aqueous solution, with a

maximum solute concentration of 1.5%. High drug content is required, in order to obtain the most nearly spherical particles. This entails shrunken and wrinkled particle morphology. Further, after suspension in butanol, to facilitate Coulter analysis, sonication is apparently necessary, implying that the particles are not fully dry.

It is an object behind the present invention to provide a therapeutic delivery vehicle and a composition that are better adapted than products of the prior art, for delivery to the alveoli in particular.

Summary of the Invention

According to the present invention, it has surprisingly been found that, in microparticles (and also microcapsules and microspheres) that are also suitable as an intermediate product, i.e. before fixing, in the production of air-containing microcapsules for diagnostic imaging, e.g. as disclosed in WO-A-9218164 as "intermediate microcapsules", the wall-forming material is substantially unaffected by spray-drying. Thus, highly uniform microparticles, microspheres or microcapsules of heat-sensitive materials such as enzymes, peptides and proteins, e.g. HSA, and other polymers, may be prepared and formulated as dry powders, for therapeutic or diagnostic use.

By contrast to the prior art, it has also now been found that effective, soluble carriers for therapeutic and diagnostic agents can be prepared, by spray-drying, and which are free-flowing, smooth, spherical microparticles of water-soluble material, e.g. human serum albumin (HSA), having a mass median particle size of 1 to 10 μm. More generally, a process for preparing microcapsules of the invention comprises atomising a solution (or dispersion) of a wall-forming material. A therapeutic or diagnostic agent may be atomised therewith, or coupled to the microcapsules thus produced. Alternatively, the material may be an active agent itself. In particular, it has been found that, under the conditions stated herein, and more

generally described by Sutton et al (1992), e.g. using an appropriate combination of higher solute concentrations and higher air:liquid flow ratios than Haghpanah et al, and shell-forming enhancers, remarkably smooth spherical microparticles of various materials may be produced. The spherical nature of the microparticles can be established by means other than mere maximum size analysis, i.e. the laser light diffraction technique described by Haghpanah et al. Moreover, the particle size and size distribution of the product can be controlled within a tighter range, and with greater reproducibility. For example, by Coulter analysis, 98% of the particles can be smaller than 6 μm on a number basis, within an interquartile range of 2 μm, and with less than 0.5 μm mean size variation between batches. Furthermore, when tested in a dry powder inhaler under development, reproducible dosing was achieved, and subsequent aerosolisation under normal flow conditions (30 l/min) resulted in excellent separation of microparticles from excipient.

Unfixed capsules of this invention, composed of non-denatured HSA or other spray-dryable material, possess highly smooth surfaces and may be processed with relatively low levels of excipients to produce free-flowing powders ideal for dry powder inhalers. Using this approach, it is possible to produce heterogeneous microcapsules which are comprised of a suspending excipients and active principle. This has the advantage of yielding free-flowing powder of active principles which may be further processed to give powders that dose and aerosolise with excellent reproducibility and accuracy.

In addition, the process of spray-drying, in its current form, gives rise to relatively little denaturation and conversion to polymers in the production of the free-flowing powder. In all cases, the size of the microcapsule suspension can be such that 90% of the mass lies within the desired size, e.g. the respirable region of 1-5 μm.

In essence therefore we have defined how to produce microparticles which are: predominantly 1-5 μm in size; smooth and spherical; gas-containing; and composed of undamaged protein molecules and which may be stored and shipped prior to other processing steps. In preparing intermediate microcapsules for ultrasound imaging, we have defined those characteristics of a process and the resulting powder which are essential for the production of superior powders for dry powder inhalers (DPI's). We find that many of the assays which have been developed for the echocontrast agent are suitable for defining those parameters of the particles which are advantageous for DPI powders, namely; echogenicity and pressure resistance of cross-linked particles defining perfectly formed microparticles; microscopic evaluation in DPX or solvents, defining sphericity and gas-containing properties of soluble intermediate capsules; size and size distribution analysis and also the monomeric protein assay to define the final level of fixation of the product.

Especially for use in therapy, considerable care is necessary in order to control particle size and size distribution. We have chosen a biocompatible polymer which when cross-linked remains innocuous and also learned how to reproducibly cross-link this molecule. In order to achieve controlled cross-linking, we have divorced the processes of microparticle formation and cross-linking which other emulsion and solvent evaporation process do not. This means that the initial step of the process does not damage the wall-forming material. We have defined the particular parameters which are important for complete particle formation and further defined more advantageous conditions which yield more intact particles. In choosing HSA as a particularly favourable polymer we have also chosen a potential carrier molecule which may: protect labile molecules; enhance uptake of peptides across the lung; bind low molecular weight drug through natural binding affinities; and be covalently modified to carry drugs

across cellular barriers to the systemic circulation and beyond.

When researchers have used spray-drying to produce microparticles of small dimensions, they have tended to use volatile solvents, which encourages rapid droplet shrinkage. Alternatively, researchers have used feedstocks with low solute content in order to keep the solution viscosity low, to enhance smaller droplet production. In both cases, when the microparticles are produced, the process has little impact on the final morphology; rather this is dictated by the components used to form the particles. We have taken the extensive learning of how to produce controlled sized particles from HSA and applied this to many other materials including active drugs. We are able to use relatively high solute contents, e.g. 10-30% w/v as opposed to 0.5-2%, to produce microparticles comprising low molecular weight active with lactose; active alone: peptides with HSA and modified polymeric carriers with active. We now find that it is the process which dictates the final particle morphology rather than the composition of the solutes. Further, we are able to use combinations of aqueous and water-miscible solvents to enhance particle morphology. Thus we have a "process" driven methodology which allows beneficial production of smooth, spherical controlled sized particles suitable for pulmonary delivery.

It has been found that the process of the invention can be controlled in order to obtain microspheres with desired characteristics. Thus, the pressure at which the protein solution is supplied to the spray nozzle may be varied, for example from 1.0-10.0 x 10^5 Pa, preferably 2-8 x 10^5 Pa, and most preferably about 7.5 x 10^5 Pa. Other parameters may be varied as disclosed below. In this way, novel microspheres may be obtained.

A further aspect of the invention provides hollow microspheres in which more than 30%, preferably more than 40%, 50%, or 60%, of the microspheres have a diameter

within a 2 μm range and at least 90%, preferably at least 95% or 99%, have a diameter within the range 1.0-8.0 μm.

The interquartile range may be 2 μm, with a median diameter of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 μm.

Thus, at least 30%, 40%, 50% or 60% of the microspheres may have diameters within the range 1.5-3.5 μm, 2.0-4.0 μm, 3.0-5.0 μm, 4.0-6.0 μm, 5.0-7.0 μm or 6.0-8.0 μm. Preferably a said percentage of the microspheres have diameters within a 1.0 μm range, such as 1.5-2.5 μm, 2.0-3.0 μm, 3.0-4.0 μm, 4.0-5.0 μm, 5.0-6.0 μm, 6.0-7.0 μm or 7.0-8.0 μm.

A further aspect of the invention provides hollow microspheres with proteinaceous walls in which at least 90%, preferably at least 95% or 99%, of the microspheres have a diameter in the range 1.0-8.0 μm; at least 90%, preferably at least 95% or 99%, of the microspheres have a wall thickness of 40-500 nm, preferably 100-500 nm.

Description of the Invention

The wall-forming material and process conditions should be so chosen that the product is sufficiently non-toxic and non-immunogenic in the conditions of use, which will clearly depend on the dose administered and duration of treatment. The wall-forming material may be a starch derivative, a synthetic polymer such as tert-butyloxy-carbonylmethyl polyglutamate (US-A-4888398) or a polysaccharide such as polydextrose.

Generally, the wall-forming material can be selected from most hydrophilic, biodegradable physiologically compatible polymers, as described in more detail in WO-A-9218164.

Preferably, the wall-forming material is proteinaceous. For example, it may be collagen, gelatin or (serum) albumin, in each case preferably of human origin (i.e. derived from humans or corresponding in structure to the human protein). Most preferably, it is human serum albumin (HSA) derived from blood donations or, ideally, from the fermentation of microorganisms (including cell

lines) which have been transformed or transfected to express HSA. Further detail is given in WO-A-9218164.

The protein solution or dispersion is preferably 0.1 to 50% w/v, more preferably about 5.0-25.0% protein, particularly when the protein is albumin. About 20% is optimal. Mixtures of wall-forming materials may be used, in which case the percentages in the last two sentences refer to the total content of wall-forming material.

The preparation to be sprayed may contain substances other than the wall-forming material and solvent or carrier liquid. Again, reference may be made to WO-A-9218164.

The protein solution or dispersion (preferably solution), referred to hereinafter as the "protein preparation", is atomised and spray-dried by any suitable technique which results in discrete microspheres or microcapsules of 1 to 10 μm diameter. These figures refer to at least 90% of the population of microcapsules, the diameter being measured with a Coulter Master Sizer II. The term "microcapsules" means hollow particles enclosing a space, which space is filled with a gas or vapour but not with any solid materials. Honeycombed particles resembling the confectionery sold in the UK as Maltesers® are not formed.

The atomising comprising forming an aerosol of the protein preparation by, for example, forcing the preparation through at least one orifice under pressure into, or by using a centrifugal atomiser in a chamber of warm air or other inert gas. The chamber should be big enough for the largest ejected drops not to strike the walls before drying. The gas or vapour in the chamber is clean (i.e. preferably sterile and pyrogen-free) and non-toxic when administered into the bloodstream in the amounts concomitant with administration of the microcapsules in use. The rate of evaporation of the liquid from the protein preparation should be sufficiently high to form hollow microcapsules but not so high as to burst the microcapsules. The rate of evaporation may be controlled

by varying the gas flow rate, concentration of protein in the protein preparation, nature of liquid carrier, feed rate of the solution and, more importantly, the temperature of the gas encountered by the aerosol. With an albumin concentration of 15-25% in water, an inlet gas temperature of at least about 100°C, preferably at least 110°C, is generally sufficient to ensure hollowness and the temperature may be as high as 250°C without the capsules bursting. About 180-240°C, preferably about 210-230°C and most preferably about 220°C, is optimal, at least for albumin. Since the temperature of the gas encountered by the aerosol will depend also on the rate at which the aerosol is delivered and on the liquid content of the protein preparation, the outlet temperature may be monitored to ensure an adequate temperature in the chamber. An outlet temperature of 40-150°C has been found to be suitable. Controlling the flow rate has been found to be useful in controlling the other parameters such as the number of intact hollow particles.

The microcapsules comprise typically 96-98% monomeric HSA.

More particularly, microparticles of the invention preferably have a maximum interquartile range of 3 μm, more preferably 2 μm, and most preferably 1.5 μm, with respect to their mass median particle size. The mass median particle diameter is determined by Coulter counter with a conversion to a volume-size distribution. This is achieved by spray-drying in which there is a combination of low feed stock flow rate with high levels of atomisation and drying air. The effect is to produce microcapsules of very defined size and tight size distribution.

Several workers have designed equations to define the mean droplet size of pneumatic nozzles; a simple version of the various parameters which affect mean droplet size is as follows:

$$D = A/(V^2.d)^a + B.(M_{air}/M_{liq})^{-b}$$

Where D = Mean droplet size
A = Constant related to nozzle design
B = Constant related to liquid viscosity
V = Relative air velocity between liquid and nozzle
d = Air density
M_{air} and M_{liq} = Mass of air and liquid flow
a and b = Constants related to nozzle design

Clearly, for any given nozzle design, the droplet size is most affected by the relative velocity at the nozzle and concurrently the mass ratio of air to liquid. For most common drying use, the air to liquid ratio is in the range of 0.1-10 and at these ratios it appears that the average droplet size is 15-20 μm. For the production of microparticles in the size range described herein we use an air to liquid ratio ranging from 20-1000:1. The effect is to produce particles at the high ratios which are exceedingly small by comparative standards, with very narrow size distributions. For microparticles, produced at the lower ratios of air to liquid, slightly larger particles are produced, but they still nevertheless have tight size distributions which are superior to microparticles produced by emulsion techniques.

The amount of added active principle is not critical; the microparticles may comprise at least 50, more preferably 70 or 80, and most preferably 90, % by weight HSA or other carrier material. For use in an inhaler device, the microparticles may be formulated with a conventional excipient such as lactose or glucose.

The microparticles may comprise therapeutic agent and carrier, or a compound which alone is therapeutically-active. The amount of the active principle will be chosen having regard to its nature and activity, to the mode of administration and other factors known to those skilled in the art. By way of example only, the number of particles administered may be such as to deliver 100 mg/day α-1 anti-

trypsin, or 0.1 mg/day of an active material such as beclomethasone.

The active principle may be, for example, a diagnostic substance or a classical pharmaceutical entity which may or may not bind, covalently or otherwise, to the carrier material. The therapeutic agent may be a proteinaceous material such as insulin, parathyroid hormone, calcitonin or similar bioactive peptide, albuterol, salicylate, naproxen, augmentin or a cytotoxic agent. For experimental purposes, a marker such as lysine-fluorescein may be included.

Microparticles of the invention may comprise an antagonist or receptor-binding component in addition to the therapeutic or diagnostic agent. For example, a sugar or other molecule may be included in the molecular vehicle, with a view to directing administration of the vehicle-bound drug to a given receptor at or beyond the alveoli.

HSA is used herein as an illustrative example of water-soluble carrier materials for use in the invention. Other materials that can be used include simple and complex carbohydrates, simple or complex amino- or polyamino-acids, fatty acid or fatty acid esters, or natural or recombinant human proteins or fragments or short forms thereof.

The invention allows for the nature of the dry microcapsules to be manipulated, in order to optimise the flow or vehicle properties, by changing and reducing the forces of cohesion and adhesion within the microcapsule preparation. For example, if so required, the microcapsules may be made predominantly positive or negative by the use of highly-charged monomeric or polymeric materials, e.g. lysine or poly-lysine and glutamate or poly-glutamate in systems without HSA or heterogeneous systems including HSA and active principles.

A further embodiment of the invention is the co-spray-drying of the active principle with HSA in order to facilitate stabilisation of the active principle during formulation, packing and, most importantly, during

residence on the alveolar lining. In this environment, there can be intense proteolytic activity. Whilst protease inhibitors can be used to protect peptide drugs, there may well be contra-indications to this approach. By using HSA, both as excipient and vehicle, it can offer a large excess of alternative substrate on which the locally-active proteases may act. A further advantage is that, since HSA has been shown to cross the alveolar barrier, by receptor- or non-receptor-mediated transcytotic mechanisms, it may be used as a vehicle to facilitate the passage of an active principle across the epithelial lining.

In a further embodiment, active principle may be covalently linked to HSA via cleavable linkages prior to spray-drying. This embodiment represents a method of carrying active principles all the way from device to bloodstream, and possibly to targets within the body. The formation of particles with optimal aerodynamic size means that the "physical" vehicle delivers the active principle to the site of absorption. Once deposited upon the alveoli, the "molecular" vehicle then protects and facilitates passage into the bloodstream and, once in the bloodstream, can further enhance circulatory half-life and even direct the active principle to certain sites within the body on the basis of receptor-mediated events.

A suitable linker technology is described in WO-A-9317713 (Rijksuniversiteit Groningen). Esterase-sensitive polyhydroxy acid linkers are described. Such technology, used in the derivatisation of HSA prior to spray-drying, enables the production of a covalent carrier system for delivery of drugs to the systemic vasculature. This utilises the potential of HSA to cross the alveoli to carry drugs over a prolonged period whilst protecting potentially unstable entities.

Although the active principle used in this invention may be imbibed into or otherwise associated with the microparticles after their formation, it is preferably formulated with the HSA. The microparticles may be at

least partly coated with a hydrophobic or water-insoluble material such as a fatty acid, in order to delay their rate of dissolution and to protect against hydroscopic growth.

The following Examples illustrate the invention. The spray dryer used in the Examples, available from A/S Niro Atomizer, Soeborg, Denmark, under the trade name "Mobile Minor", is described in detail in WO-A-9218164.

Example 1

A 20% solution of sterile, pyrogen-free HSA in pyrogen-free water (suitable for injection) was pumped to the nozzle of a two-fluid nozzle atomizer mounted in the commercial spray-drying unit described above. The peristaltic pump speed was maintained at a rate of approximately 10 ml/minute such that with an inlet air temperature of 220°C the outlet air temperature was maintained at 95°C.

Compressed air was supplied to the two fluid atomising nozzle at 2.0-6.0 Bar (2.0-6.0 x 10^5 Pa). In this range microcapsules with a mean size of 4.25-6.2 μm are obtained.

Typically an increase in mean particle size (by reduced atomisation pressure) led to an increase in the amount of microcapsules over 10 μm in size (see Table 1).

Table 1

Effects of Atomisation Pressure on Frequency of Microcapsules Over 10 μm in Diameter

Atomisation Pressure ($x10^5$ Pa)	% Frequency over 10 mm
6.0	0.8
5.0	3.3
3.5	6.6
2.5	8.6
2.0	13.1

Under the conditions described above, i.e. the first step of Example 1 of WO-A-9218164, with a nozzle pressure

of 7.5 bar, microparticles were produced with a particle size of 4.7 μm. These soluble microparticles were smooth and spherical with less than 1% of the particles over a particle size of 6 μm. The microparticles were dissolved in aqueous medium and the molecular weight of the HSA determined by gel filtration chromatography. The resultant chromatograms for the HSA before and after spray-drying HSA are essentially the same. Further analysis of the HSA before and after spray-drying by means of tryptic peptide mapping with HPLC revealed that there were no observable differences in the peptides liberated. Both analyses show that, under the conditions of spray-drying described to produce microparticles of 4.7 μm, little or no structural damage is done to the protein.

Example 2

Alpha-1 antitrypsin derived from human serum was spray-dried under conditions similar to Example 1 with an inlet temperature of 150°C and an outlet temperature of 80°C. In all other respects the conditions for drying were the same as Example 1. The soluble microparticles produced had a mean size of 4.5 μm. The microparticles were dissolved in aqueous medium and analysed for retention of protein structure and normal trypsin inhibitory activity, then compared to the original freeze dried starting material. Analysis by gel permeation and reverse phase chromatography and capillary electrophoresis, revealed that there were no significant structural changes after spray-drying. Analysis of the inhibitory activity (Table 2) showed that within the experimental error, full retention of inhibitory activity had been achieved.

Table 2

Run Number	Percentage of Activity Retained
1	84
2	222
3	148

Example 3

Using the general method of Example 1, microcapsules composed of alcohol dehydrogenase (ADH) and lactose were prepared (ADH 0.1% w/w; Lactose 99.9% w/w). We find that optimisation of the spray-drying step is required to maximise the retention of enzyme activity. The general conditions of Example 1 were used, but the inlet and outlet temperature were changed to give conditions which allowed us to produce microparticles of the desired size (4-5 μm) that retained full activity after drying and reconstitution in aqueous media. The percentage of activity retained compared with the original material is shown for each of the spray-drying conditions shown (Table 3). The microcapsules were smooth and spherical and contained air as evidenced by their appearance in diphenylxylene (DPX) under light microscopy.

Table 3

Run	Inlet Temp. °C	Outlet Temp. °C	Activity Remaining (%)
1	220	73	57
2	175	71	98

Example 4

A series of experiments was performed under the conditions described in Example 1, to examine the influence of liquid feed rate on the yield of intact spherical particles. We find that, using the ability of gas-containing microparticles to reflect ultrasound, we are able to determine optimal condition for maximising the yield of intact smooth spherical microcapsules. The microparticles formed after spray-drying are heat-fixed, to render them insoluble, and then suspended in water to make the echo measurements. We find that increasing the liquid feed rate decreases the number of intact microparticles formed during the initial spray-drying (Table 4). The mean particle size and overall pressure stability, i.e.

thickness of the shell, do not change but the total echogenicity does, as the liquid flow rate is increased from 4 to 16 ml/min. We find that slower rates of evaporation (at higher liquid flow rates) lead to fewer intact spherical particles being formed.

Table 4

Flow Rates (ml/min)	4	8	12	16
Mean size (μm)	3.08	3.04	3.13	3.07
Echogenicity (video density units)	22	21	14	10
Echogenicity after pressure (video density units)	20	18	10	8

The assay was conducted by resuspending the heat-fixed microparticles at a concentration of $1x10^6$ ml in 350 ml of water. This solution is stirred slowly in a 500 ml beaker above which is mounted an 3.5 MHz ultrasound probe attached to a Sonus 1000 medical imaging machine. The grey scale images obtained are captured by an image analyser and compared against a water blank to yield video density units of echo reflectance. The assay can also be adapted to examine the pressure resistance, by assessing the echo-reflectance before and after exposure of the sample to cyclical bursts of pressure applied to the stock solution of particles. This analysis distinguishes incomplete particles which entrain air upon reconstitution, from fully spherical particles which "encapsulate" air within the shell. Incomplete particles do not show pressure resistance and lose the ability to reflect ultrasound immediately. The dose response for fixed albumin particles of Example 1 is c. 5, 9, 13, 20, 22 and 24 VDU's (backscatter intensity) at respective microcapsule concentrations of 0.25, 0.5, 1, 2, 3 and 4 x 10^6 per ml.

Example 5

Significant experimentation to reduce the particle size and narrow the size distribution has been performed. This was pursued to effectively increase the gas content of

the echocontrast agent and reduce the number of oversized particles. This exercise is also beneficial to respiratory formulations in that it maximises the potential number of respirable particles in the 1-5 μm range and produces inherently more smooth particles which will be less cohesive than non-spherical particles of similar size.

We find that it is possible to reduce particle size by lowering the solutes content of the feedstock. This effect, is in part, mediated by the effects of viscosity on droplet formation. However, we also find that by lowering the solute content under the conditions we use leads to a significant decrease in the number of intact particles. By further experimentation we have found that incorporation of water-miscible, volatile solvents in the feedstock, significantly increase the rate of shell formation during drying, with a concomitant increase in the number of intact particles or hollow particles (Table 5). The assessment of hollowness is made by microscopic evaluation of particles floating to the surface of the coverslip on a haemocytometer versus particle count by Coulter counting.

Table 5

Run	HSA Content of Feedstock (%)	Ethanol Content of Feedstock (%)	Mean Particle Size (μm)	Percentage of Hollow Particles (%)
1	10	0	3.7	12.5
2	10	25	3.52	64.3

Example 6

A range of materials has been used to manufacture smooth spherical soluble microparticles. The range of microparticles produced includes inert materials such as HSA, lactose, mannitol, sodium alginate; active materials alone such as α1-antitrypsin; and mixtures of active and inert carrier such as lactose/alcohol dehydrogenase, lactose/budesonide, HSA/salbutamol. In all cases, smooth, spherical and gas-containing particles are produced.

We have assessed the success of the process in maintaining control over the morphology of the particles. The particles are suspended in propanol and then visualised by microscopy. Those particles which contain gas appear to have an intense white core surrounded by an intact black rim whilst broken or miss-formed particles appear as ghosts. Microscopic evaluation of the following microparticles exemplifies the range of materials and actives which can be dried to produce smooth spherical particles:

HSA
Casein
Haemoglobin
Lactose
ADH/lactose
HSA/Peroxidase
Lactose/Salbutamol
Lactose/Budesonide

Example 7

Lactose and Budesonide were spray-dried under the conditions described in the table below (Table 6).

Table 6

Parameter	Setting
Inlet Temperature	220°C
Outlet Temperature	85°C
Atomisation Pressure	7.5 bar
Damper Setting	0.5
Feed Rate	3.88 g/min
Stock Solution	9.3% w/v Budesonide, 85%v/v Ethanol 19% w/v lactose

The resultant dry powder was blended with excipient grade lactose in a V type blender in the proportions outlined in Table 7. The blends were then loaded into gelatin capsules and discharged from a Rotahaler™ into a twin stage impinger run at 60 l/min. The respirable fraction was calculated as the percentage deposited into the lower chamber.

Table 7

Formulation Number	% Budesonide in spray dried particles	% spray dried product in blend	% fast flow lactose in blend	Respirable fraction
1	9.3	10	90	42
2	9.3	15	85	29
3	9.3	20	80	34
4	5.7	30	70	36

The respirable fractions obtained are considerably superior to micronised product currently used in this device which are usually in the range of 10-20% maximum.

The Budesonide/Lactose formulations detailed in Example 7 were tested in an experimental gravity fed multi-dose DPI. The parameters examined were the variation of emitted dose over 30 shots and the respirable fraction in a four-stage impinger device. The results are shown below (Table 8).

Table 8

Formulation Number	Dose (mg)	Fine Particle (Respirable) Fraction (%)	CofV on Emitted Dose (%)
1	4	52	2.0
2	4.2	42	2.8
3	3.7	58	8.1

For current DPI devices, the preliminary US Pharmacopoeia recommendation appears likely to be less than 25% variation in the emitted dose. Clearly in all of the formulations tested so far we are within the current specifications and in the case of formulations 1 and 2 we are significantly under the current limits.

Example 8

To decrease the dissolution rate of soluble microcapsules as described in preceding Examples, microcapsules may be coated with fatty acids such as palmitic or behenic acids. The soluble microcapsules of

Example 1 were coated by suspending a mixture of soluble HSA microcapsules and glucose (50% w/w) in an ethanolic solution containing 10% palmitic or behenic acid. The solution was evaporated and the resultant cake milled by passage through a Fritsch mill.

The efficacy of coating was assessed by an indirect method derived from our previous ultrasound studies. Ultrasound images were gathered from a beaker of water containing 1 x 10^6 microcapsules/ml using a HP Sonus 1000 ultrasound machine linked to an image analyser. Video intensity over a blank reading (VDU) was measured over time (Table 9).

The uncoated microcapsules very quickly lost all air and thus the potential to reflect ultrasound. However, coated microcapsules retained their structure for a longer period and hence showed a prolonged signal over several minutes.

Table 9

Echogenicity of Coated HSA Microcapsules

Time (min)	Echogenicity (VDU)		
	HSA only	HSA/Palmitic Coated	HSA/Behenic Coated
0	1.75	1.91	0.88
5	0.043	0.482	0.524
10	0	0	0.004

Example 9

Soluble mannitol microcapsules were prepared as set out in Example 1 (15% aqueous mannitol spray-drying feedstock) and coated with palmitic acid and behenic acid as described in Example 8. A sample of each was suspended in water and the echogenicity measured. Ten minutes after the initial analysis, the echogenicity of the suspended samples was repeated (Table 10).

Table 10

Echogenicity of Coated Mannitol Microcapsules

Time (min)	Echogenicity (VDU)		
	Mannitol	+ Palmitic	+ Behenic
0	1.6	1.7	0.92
10	0.33	0.5	0.24
17	0	0.84	0

Example 10

Soluble microcapsules with a model active (Lysine-Fluoroscein) contained within the matrix were prepared to allow the production of a free-flowing dry powder form of the "active" compound. On dissolution of the microcapsules, the active compound was released in its native form.

Using lysine as a model compound, the molecule was tagged with fluorescein isothiocyanate (FITC) to allow the compound to be monitored during the preparation of the soluble microcapsules and the subsequent release during dissolution.

3 g of lysine was added to FITC (0.5 g total) in carbonate buffer. After one hour incubation at 30°C, the resultant solution was tested for the formation of the FITC-lysine adduct by TLC. This showed the presence of a stable FITC-lysine adduct.

The FITC-lysine adduct was mixed with 143 ml of 25% ethanol containing 100 mg/ml HSA to give the spray-drying feedstock. The spray-drying conditions used to form the microcapsules are detailed in Table 11 below. In the absence of ethanol we have found that only a small percentage of the particles are smooth and spherical.

The spray-drying process produced 17.21 g of microcapsules that did not dissolve when a sample was resuspended in ethanol. Moreover, no release of the FITC-lysine adduct was observed. However, when 10 ml water was added to the ethanol-suspended microcapsules, the

microcapsules dissolved and the FITC-lysine was released. Analysis of the adduct using TLC before incorporation into the microcapsules and after release from the microcapsules on dissolution showed the model compound was unchanged.

Table 11

Spray-Drying Conditions

Parameter	Setting
Inlet Temperature	220°C
Outlet Temperature	85°C
Atomisation Pressure	7.5 bar
Damper Setting	0.5
Feed Rate	3.88 g/min
Stock Solution	25% v/v Ethanol, 10% w/v HSA

The soluble microcapsules were sized in a non-aqueous system of ammonium thiocyanate and propan-2-ol using a Multisizer II (Coulter Electronics). The microcapsules had a mean size of 3.28 ± 0.6 μm and with 90% of the mass within 2-5 μm.

The microcapsules were mixed with glucose (50% w/w microcapsules : 50% w/w glucose), and milled by the passage of the mixture through a Fritsch mill three times. When a sample of the powder was added to water, the FITC-lysine was released intact when compared with its original form as determined by TLC analysis. This example shows the feasibility of making an amino acid or peptide formulation which could be used for respiratory formulations, which incorporates HSA within the formulation.

Example 11

500 mg beclomethasone was dissolved in ethanol and added to 50 ml HSA feedstock (10% w/v) and spray-dried using the conditions outlined in Example 10. The microcapsules hence formed were sized in the non-aqueous system as detailed in Example 10. The microcapsules had a mean size of 3.13 ± 0.71 μm, 90% of which were between 2 and 5 μm.

The beclomethasone was extracted from the microcapsules by the precipitation of the HSA in 10% TCA, and the supernatant was extracted into ethanol. The ethanol extract was analysed using HPLC, at a wavelength 242 nm. The beclomethasone detected in this extract exists in the free state, but when the albumin pellet was extracted the presence of beclomethasone bound to native HSA was observed. It was found that although the majority of the active compound was in the free state, some was present in the albumin-bound state. Since albumin partitions only slowly into the bloodstream, this allows control over the release of the active compound over an extended period of time, compared to free drug.

Example 12

Whereas in Examples 10 and 11 at least, any binding of the active compounds was an effect of the intrinsic nature of albumin, this Example gives a product following initial cross-linking of the active compound, prior to spray-drying.

To a 10 mg/ml solution of methotrexate, 25 mg carbodiimide (EDCI) was added. The solution was stirred for 4 hours to initiate and ensure complete activation of the methotrexate. 50 mg HSA was added to the activated drug and stirred for 3 hours at room temperature. The methotrexate is chemically bound to the HSA via the amine groups on the albumin. This conjugate was then used as the spray-drying feedstock as detailed in Example 10.

The soluble microcapsules thus made were sampled, characterised and analysed for drug content. The microcapsules had a mean size of 3.2 ± 0.6 μm with 90% by mass between 2-5 μm. The analysis of the drug content of the microcapsules showed that the microcapsules did not release drug; even after dissolution, drug was still bound to the HSA. Proteinase K digestion of the albumin released the bound drug which was shown to be linked to only a limited number of amino-acids and small peptides. It has been shown previously that the activity of doxorubicin

bound to polymeric carriers proves beneficial in tumours, showing the multidrug-resistant phenotype.

Example 13

Naproxen microcapsules were prepared as detailed in Examples 10 and 12 using a ratio of 1 to 5, drug to HSA. The soluble microcapsules retained the active compound of a non-aqueous solvent. Moreover, on dissolution of the microcapsules in aqueous solution, the active compound was still bound to the albumin, as shown by HPLC analysis at 262 nm, as before. The naproxen was released from the albumin on digestion with proteinase K and esterases.

Example 14

Using samples of the microcapsules produced in Examples 8 to 13, an assessment of their behaviour in a dry powder inhaler was made. The dosing reproducibility of each formulation was assessed in conjunction with the aerolisation of the sample by microscopic evaluation.

A sample of each formulation was added to the storage funnel of an experimental dry powder inhaler (DPI). The dry powder inhaler used pressurised air to force the powder into a dosing measure. The dosing measure used was calibrated using spray-dried lactose.

Although the amounts dispensed into the dosing measure varied between samples as a function of their composition, the dosing reproducibility for each sample was very consistent; with a mean of 5.0 ± 0.25 mg obtained for three dosing trials.

The aerolisation behaviour of the samples was tested by connecting the inhaler to a vacuum chamber; simulated inhalation was achieved by the release of the vacuum through the DPI and collection of the airborne dose was made on resin coated microscope slides. These slides were evaluated for dispersion of the particles. The slides showed that the DPI had deagglomerated the samples forming an even dispersion of microparticles on the microscope slides.

Example 15

The performance of the dry powder formulations from Examples 10 to 13 was analysed using the twin impinger method (Apparatus A for pressurised inhalations, British Pharmacopoeia 1988) following discharge from a Rotahaler (Glaxo UK) with 7 ml in stage 1 and 30 ml in stage 2 of distilled water. The formulations were delivered from size 3 gelatin capsules using a Rotahaler attached to the twin impinger using a rubber adapter. The vacuum pump was operated at 60 l/min for two 3 second bursts. The amount of each sample reaching stage 1 and stage 2 levels of the impinger was analysed. All samples showed the largest percentage deposition to occur in stage 2 of the impinger indicating optimal sized particles for alveoli delivery.

Example 16

A comparison of the dosing and deposition of fixed insoluble microcapsules and soluble microcapsules as produced in Example 10 was made in the lung of rabbits.

Anaethestised New Zealand white rabbits were dosed either with soluble microcapsules or fixed microcapsules. The dosing was carried out using a computer controlled nebuliser (Mumed Ltd., UK). The soluble microcapsules were suspended in CFC 11 and the fixed particles were suspended in water. After dosing, the lungs of the rabbits were removed and an assessment of the deposition of the capsules made.

The fixed capsules were found intact in the alveoli tissue of the lung. This showed that the microcapsules were of the appropriate size for dispersion through the lungs. In comparison, no evidence of the presence of intact soluble microcapsules was found, the capsules having dissolved in the fluids of the lung. However, the presence of FITC-lysine adduct was observed in some of the alveoli tissue when studied using fluorescent microscopy. In addition, the presence of the adduct was also found the blood and urine of the animals, as opposed to that of the fixed capsules which showed no presence in either.

CLAIMS:

1. Microparticles of a water-soluble material, which are smooth and spherical, and at least 90% of which have a mass median particle size of 1 to 10 μm, for use in therapy or diagnosis.

2. Microparticles of a water-soluble material, which are smooth and spherical, and at least 90% of which have a mass median particle size of 1 to 10 μm which carry a therapeutic or diagnostic agent.

3. Microparticles according to claim 2, obtainable by spray-drying an aqueous solution of said water-soluble material and the therapeutic or diagnostic agent.

4. Microparticles according to any preceding claim, wherein said particle size is 1 to 5 μm.

5. Microparticles according to any preceding claim, which have a maximum interquartile range of 3 μm.

6. Microparticles according to claim 5, which have a maximum interquartile range of 2 μm.

7. Microparticles according to any preceding claim, which are sterile.

8. Microparticles according to any preceding claim, which are at least partly coated with a water-insoluble material.

9. Microparticles according to any preceding claim, which additionally carry a receptor-binding component.

10. Microparticles according to any preceding claim, wherein the water-soluble material is a carbohydrate.

11. Microparticles according to any of claims 1 to 9, wherein the water-soluble material is an amino- or polyamino-acid.

12. Microparticles according to any of claims 1 to 9, wherein the water-soluble material is a fatty acid or ester thereof.

13. Microparticles according to any of claims 1 to 9, wherein the water-soluble material is a protein, peptide or enzyme.

14. Microparticles according to claim 13, wherein the water-soluble material is a human protein or fragment, in natural or recombinant form.

15. Microparticles according to claim 14, wherein the water-soluble material is human serum albumin.

16. Microparticles according to any preceding claim, wherein the water-soluble material is chemically or enzymatically modified, prior to formation of the microparticles.

17. An inhaler device adapted to deliver a therapeutic agent via the pulmonary airways, which comprises the therapeutic agent in the form of microparticles according to any preceding claim.

18. Use of a therapeutic agent for the manufacture of a medicament for treatment of a complaint on which the therapeutic agent acts on administration via the pulmonary airways, characterised in that the therapeutic agent is in the form of microparticles according to any of claims 1 to 16.

19. In a method of treating a complaint by administration to the patient of an effective amount of a therapeutic agent that acts via pulmonary airways to treat the complaint, the improvement comprising administration of the therapeutic agent in the form of microparticles according to any of claims 1 to 16.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/02279

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/16 A61K9/50 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 611 567 (TEIJIN LIMITED) 24 August 1994 see page 5, line 3 - line 11 see page 6, line 26 - line 49 see example 1 ---	1-4,10, 11,17-19
X	EP,A,0 606 486 (TEIJIN LIMITED) 20 July 1994 see claims 1,11,12 see page 6, line 32 - page 8, line 32 see page 8, line 53 - page 9, line 5 --- -/--	1-4,10, 11,17-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 1996	19.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ventura Amat, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, vol. 40, no. 4, August 1994 STUTTGART, DE, pages 203-208, XP 000459725 CONTE, U., ET AL. 'Spray dried Albumin Microspheres containing Nicardipine' see the whole document	1,14,15, 17-19

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]nal Application No
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0611567	24-08-94	AU-B-	659328	11-05-95
		AU-B-	4355693	04-01-94
		AU-B-	660824	06-07-95
		AU-B-	4355593	04-01-94
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		CA-A-	2115444	23-12-93
		EP-A-	0606486	20-07-94
		WO-A-	9325193	23-12-93
		WO-A-	9325198	23-12-93
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		CA-A-	2115444	23-12-93
		EP-A-	0611567	24-08-94
		WO-A-	9325193	23-12-93
		WO-A-	9325198	23-12-93

EXHIBIT C

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(54) Title: CROSS-LINKED MICROPARTICLES AND THEIR USE AS THERAPEUTIC VEHICLES

(57) Abstract

A sterile powder comprising microparticles, 0.1 to 50 μm in diameter, obtainable by spray-drying and cross-linking a water-soluble material having free functional groups, is characterised in that the microparticles are hydrophilic, can be reconstituted in water to give a monodisperse suspension, and have retained said groups available for derivatisation. The particles are linked to drugs or other functional molecules, and used as vehicles in therapy.

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CROSS-LINKED MICROPARTICLES AND THEIR USE AS THERAPEUTIC VEHICLES

Field of the Invention

This invention relates to cross-linked microparticles and their use as therapeutic vehicles.

Background of the Invention

Microparticulate carrier systems are increasingly attracting attention for use in the parenteral delivery of therapeutic and diagnostics agents. A plethora of microparticle technology systems and chemistries has been proffered as vehicles to deliver agents subcutaneously, intravenously and intra-arterially. There are several key aspects to the "ideal vehicle". These include size, size distribution, payload, rate of biodegradation, ease of use, release kinetics and scalable reproducible production. Individual aspects of this "ideal vehicle" have been successfully addressed by others, notably drug payload, rate of biodegradation and, in part, size and size distribution.

Known vehicles have been manufactured by various techniques, largely solvent and emulsion-based. A disadvantage of these methods is that control of the key elements of the vehicle was attempted within one or two steps of the production. Thus, size, size distribution, payload and rate of biodegradation were all imparted on the product in a single, dynamic environment, typified by single and double emulsion systems or solvent evaporation techniques. Typically, for emulsion processes, the solution of drug, polymer and surface-modifying agents has been mixed with an insoluble solvent, emulsified, heated or stabilised to fix the particles, and then cleansed to remove oils or solvent incompatible with parenteral use.

The reaction vessel in emulsion or solvent evaporation systems is a principal characteristic of the prior art techniques. Within this vessel, the control of the microparticle morphology is achieved by balancing the interfacial forces of oil and water components, the

interaction of solute at the interface, the balance between agitation, heat and shell formation and, of course, the incorporation of active within the polymer matrix. However, such technologies are largely incompatible with large-scale pharmaceutical manufacture required for a parenteral agent.

Almost without exception, the control of size and size distribution of known microparticles was vastly inferior to the size control attained by the spray-drying techniques described in the PCT publications WO-A-9112823, WO-A-9218164 and WO-A-9408627, in the production of microparticles for use in echo-contrast imaging and other potential parenteral uses. The acute toxicity of intravenous microparticles is largely associated with capillary blockage in the pulmonary circulation, concurrent decrease in the pulmonary venous pressure and loss of compliance. The relationship between particle size and LD toxicity is well recorded. Our own data show the precipitous elevation in toxicity of iv particles, with a mean size in excess of 6 μm, the notional capillary size in lung tissue for non-deformable microcapsules.

Typically, the larger the mean size of the capsules, the significantly broader their size distribution, and the range of microparticle sizes can span two orders of magnitude. For therapeutic use, such as chemo-embolisation, the prospect of injecting a microparticle preparation containing particles ranging in size from 5-100 μm is largely inconsistent with the concept of highly regionalised targeted delivery. At the upper end, there is the prospect of embolising major vessels up to and above 100 μm in diameter, with the attendant risk of necrosing large perfusion territories; at the smaller end of the range, what essentially amounts to systemic distribution becomes possible.

The mechanism by which sustained release has previously been most commonly achieved in microparticle systems has been the control of matrix erosion and release

to the surrounding medium of embedded or imbibed actives. The actives have either been incorporated at the time of particle production or imbibed into the matrix following fixation or stabilisation.

The incorporation of drugs into the matrix of the known microcapsules required heating in the presence of water and, inevitably, oxygen. This would almost certainly lead to adulteration of the drug by oxidative damage or uncontrolled cross-linking to the vehicle. In those cases where chemical stabilisation is used, the potential loss of active would be even worse.

Another mechanism of slowing or modifying release rates of drugs from soluble polymeric carriers has been to link the actives *via* covalent linkages to the soluble polymer. In general, this has not been applied to microparticulate systems where drugs, ligands or antibodies are linked to particulate carriers.

The main impediment to linking actives to prior art microparticles, is the latter's relative hydrophobicity. Since many of the chemical reactions required to achieve linkage are carried out in aqueous medium, such hydrophobic microparticles are almost impossible to derivatise. Where previous workers have produced hydrophilic microcapsules, they required complex formation in the presence of hydrophilic polymer, in an emulsion process.

The rate of biodegradation of microcapsules is determined largely by the extent of cross-linking. In the prior art systems, changes in cross-linking have detrimental effects upon drug loading and the ability subsequently to formulate the microparticles. Little effort was expended in attempting to manipulate this parameter to control the rate of biodegradation and drug release.

Microparticles of the prior art have required significant amounts of surfactants or sonication to achieve monodispersed suspension in aqueous media. Even when reconstituted, the microparticles have a propensity to

agglomerate and are thus difficult to administer through hypodermic syringes.

Summary of the Invention

This invention is based on the discovery that microparticles of the type described in the PCT publications WO-A-9112823, WO-A-9218164 and WO-A-9408627, having good particle characteristics, can retain, even after thermal cross-linking, their hydrophilic properties and the ability to be reconstituted in water to give a monodisperse suspension. Further, functional groups such as carboxyl, amine, hydroxyl or sulfhydryl groups in the starting material are retained, and are available for derivatisation.

According to the present invention, a sterile powder comprises smooth, spherical microparticles, 0.1 to 50 μm in diameter, of cross-linked materials, the microparticles being hydrophilic and capable of reconstitution in water to give a mono-disperse suspension, and which additionally comprises a physiologically or diagnostically-active component linked directly or indirectly to microparticles *via* free functional, e.g. amine, hydroxyl, carboxyl or sulfhydryl, groups thereon.

Description of the Invention

This invention preferably utilises a microparticle production technique of the type described in the PCT publications, *supra* (the contents of which are incorporated herein by reference), in which there is tight control over size, size distribution, payload, rate of biodegradation and release kinetics, providing ease of use and scalable production. The microparticles of this invention may be tailored at will to suit the application whilst retaining, in all cases, the ability to produce the loaded vehicle at scale to high levels of pharmaceutical practice and always with the same level of control. In addition to control of size, independent control, in individual steps, is possible for size distribution; rate of fixation or, reciprocally, rate of biodegradation; drug loading; and formulation and

finishing. As previously disclosed in the PCT publications, *supra*, the Applicants have a fully scaled process which can produce microparticles of the nature specified. The process may be operated to pharmaceutical standards without the ingress of foreign particulates that would most certainly preclude parenteral use of microparticles produced by many of the prior art processes.

The present invention relates to the production of microparticle preparations for intravenous, intraarterial and *ex vivo* use. Intravenous particle suspensions, on reconstitution in diluent, preferably contain less than 5% by volume of particles larger than 6 μm. Furthermore, the size distribution is near Gaussian in shape, with some 50% of particles lying within a range of 5 μm, preferably 3 μm, more preferably 2 μm and most preferably 1.2 μm. A desirable distribution has 80% of particles in a range of 3 μm. (All distributions quoted on a volume or mass basis). One preferred embodiment of the invention is powders wherein 95% of the particles are smaller than 6 μm, and 80% of the particles are in the range of 1 to 6 μm, especially for iv administration. Another preferred embodiment is powders wherein 90% of the particles are smaller than 20 μm, and less than 5% by volume are smaller than 6 μm, especially for intraarterial administration.

For larger particle systems, by utilising a combination of highly controlled spray drying and a subsequent fractionation step, it is possible to produce microparticles of sufficient size and tight size distribution such that, following intraarterial administration, systemic release is eliminated and only vessels smaller than 20 μm become embolised.

In one embodiment of the current invention, we have incorporated active within the feedstock for spray drying and subsequently stabilised the particle. The advantage we have gained is vastly superior control of morphology and payload over previous methodologies.

The process used in this invention may involve the deposition of wall material and drug into dry powder state, with negligible water content, capable of being reconstituted to the original soluble components.

In this invention, cross-linking can be used to control the release rates of actives. For example, in one embodiment, the extent of cross-linking, and hence the rate of degradation, is "set" prior to attachment of the active. The active ligand may then be attached, and shows a controlled release profile determined by the extent to which the matrix was cross-linked. This has the advantage that a steady rate of release is observed without the well-known "burst" effect.

A further aspect of this invention is the manipulation of cross linking. Potential levels of derivatisable groups and temperature/time required for cross-linking may be controlled by the incorporation of additives which alter these parameters. Inclusion of lysine or polylysine, glutamate or polyglutamate, phenylalanine and tyrosine into the feedstock can have the effect of increasing or decreasing the biodegradation rate of the final microparticle preparation. Additionally, the incorporation of these additives can significantly increase the number of potential groups to which actives, or ligands, may be attached. Furthermore, incorporation of these additives can reduce the time/temperature at which microparticle stabilisation occurs during heating of the soluble microparticles formed after spray drying.

Accordingly, therefore, while the present invention provides microparticles that are especially suitable for delivery to defined sites, owing to their narrow particle size distribution, a combination of desirable features has been found that make the microparticles especially useful.

It has been discovered that microcapsules manufactured using the techniques described, have several superior properties over the prior art microparticles with regard to the ability to handle, derivatise and formulate the

microparticles. Thus, for example, the Applicants' technology enables the production of microparticles which apparently retain a substantial degree of secondary structure, and thus hydrophilicity instead of being denatured, and rendered insoluble, with hidden functional groups, as in the prior art. In particular, the microparticles:

(i) possess significant levels of amine, hydroxyl or carboxyl groups, or a combination thereof, for derivatisation,

(ii) are highly hydrophilic with full access by aqueous media for the derivatisation of the active groups,

(iii) may be manipulated in dry or wet state to yield dry final formulations that do not require surfactants or sonication to yield monodispersed suspensions of particles,

(iv) biodegrade and release active principles at a rate determined by the composition and cross-linking of the shell,

(v) may possess sizes ranging from 0.01 μm to 100 μm, and

(vi) are retained in circulation in vivo for periods of time (60 min or more), considerably longer than for the more hydrophobic known microparticles, and longer than for liposomes; opsonisation is reduced, and thrombogenic potential minimised.

The particle size is preferably below 4 μm for intravenous administration, and between 8 and 30 μm for intraarterial administration. Especially for larger particles, fractionation is an optional extra step. This particle size range can be expressed such that the ratio of the interquartile range to mean diameter is 0.2 to 0.5.

The microparticles of this invention may be derivatised by conjugation of drugs, ligands, peptides or proteins directly to the carrier using the carboxyl or

amine groups of the basic capsules or additives made to the feedstock for spray drying. For example, conjugation may be achieved using glutaraldehyde, EDCI, terephthaloyl chloride, cyanogen bromide or reductive amination. Alternatively the ligand, drug, protein or peptide may be linked *via* a biodegradable hydroxy acid linker of the kind disclosed in WO-A-9317713 (Rijksuniversiteit Groningen), the content of which is incorporated by reference.

A further advantage of this invention lies in the ability to formulate and present the product as a dry sterile powder.

Microcapsules of this invention in powder form do not have an absolute requirement for surfactants to ensure a monodispersed suspension on reconstitution. Once reconstituted, they do not agglomerate, and can be administered *via* syringe.

An aspect of the present invention is a water-compatible system manufactured from biocompatible materials. It could not be anticipated that the microparticles of the current invention could be insolubilised by heating yet retain sufficient secondary structure to remain highly hydrophilic. Evidence of the retention of secondary structure is obtained by examination of the particle isoelectric point (PI) which, at pH 4.5 to 5, is very similar to native albumin. Normally, full denaturation of albumin leads to a significant rise in PI, to a value of 6.5 to 7.0.

Further, digestion of protein microparticles with protease yields peptides which, when compared with digests of the starting soluble protein, show near identical profiles, by HPLC analogues. In addition, acid hydrolysis of protein microparticles and protein starting material show stikingly similar amino-acid content. These two analyses support the observation that the protein in the microparticles is largely native.

The novel particles are hydrophilic and have the potential to circulate for periods in excess of one hour,

offering for the first time a biocompatible carrier system showing prolonged circulatory lifetime with a highly specific affinity for ligands. The specificity of the microparticles is "set" during manufacture and imparts a high affinity ligand-binding capability normally associated with chromatography matrices or enzymes. The particles also offer potential for use in contact with biological fluids, for instance in detoxification in extracorporeal systems, bioassays on serum or blood and the separation of blood components prior to reintroduction into the body.

The following Examples illustrate the invention.

Example 1

This Example illustrates the fixation of soluble microcapsules, to form insoluble or less soluble microcapsules, by cross-linking of the shell material. The microcapsules can be cross-linked by various processes, including the use of heat or chemical means. The adjustment of the degree of fixation results in the subsequent degree to which the microcapsules will dissolve in an appropriate medium. Moreover, any active compounds bound or encapsulated within the microcapsules will be released at this point of dissolution. In addition, the degree to which the microcapsules are fixed is also reflected in the degree to which they can be digested enzymatically. The greater the degree of fixation, the greater the resistance of the microcapsules to enzymic digestion.

HSA microcapsules were produced from a spray-drying feedstock containing 150 ml of 25% ethanol containing 10.0 mg/ml HSA. The spray-drying conditions used to form the microcapsules are detailed in Table 1, below.

Table 1

Spray Dryer Condition	Setting
Inlet Temperature	220°C
Outlet Temperature-Initial	85.2°C
Outlet Temperature-Final	84.0°C
Atomisation Pressure	7.5 bar
Damper Setting	0.5
Feed Rate	3.88 g/min
Stock Solution	25% ethanol 100 mg/ml HSA

The spray-drying process produced 17.21 g of microcapsules. Microcapsules from this single production batch were divided into equal sized aliquots and heat-fixed at 175°C for 45, 55 and 75 minutes respectively. The heat fixation process renders the soluble microcapsules from the spray-drying process insoluble, by cross-linking some of the amino acids within the albumin structure. The three different heat-fixed microcapsules were sized in an aqueous system using a Multisizer II (Coulter Electronics). The microcapsules had a mean size of 3.28 ± 0.6 μm and with 90% of the mass within 2-5 μm.

Prior to binding any active component to the microcapsules, those heat-fixed for 55 minutes were analysed in various ways for their suitability as drug carriers.

Free Thiol Analysis

The free thiol group present in the albumin molecule is very susceptible to modification and hence it can be used as a measure of the state and condition of the albumin. Similarly, it should be present within the microcapsule structure providing the albumin molecule was not disrupted during formation.

Analysis of the free thiol group was carried out reacting the albumin microcapsules with DTNB, i.e. 5,5'-dithiobis(2-nitrobenzoic acid). If the free thiol is present, it reacts with the DTNB to yield a nitrobenzoic acid derivative that absorbs at 412 nm. The absorbance of

a 12 mg/ml suspension of microcapsules at 412 nm was measured. To the suspension, 50 µl of a 20% solution of DTNB in TRIS buffer was added, incubated for 10 min at room temperature and the absorbance measured. The difference between the two absorbances was calculated and from the molecular extinction coefficient of the reaction product, the concentration of the free thiol present in the microcapsules was calculated. The molecular ratio of thiol groups measured in the microcapsules was 0.4785. This compared to a value of 0.5045 for native albumin. This was not a significant difference and it was concluded that the free thiol group was unchanged during microcapsule manufacture.

The microcapsules (both soluble and insoluble) and native albumin were broken down to their constituent amino-acids by vapour phase hydrolysis using concentrated HCl at 120°C for 24 hr. The samples were then derivatised by the addition of triethylamine in 50% ethanol and followed by triethylamine and PITC in ethanol. The derivatised samples were analysed by HPLC, and the amino-acids detected at a wavelength of 254 nm.

Table 4 (at the end of the description) shows the resultant amino-acid compositions. Unexpectedly there is no significant difference between the various samples, with only very small losses of amino-acids containing carboxyl, hydroxyl and amide groups after insolubilisation of the microcapsules.

Peptide Analysis

Pepsin digestion of the microcapsules and albumin was performed using a 1 ml acidified solution of microcapsules or albumin to which was added 20 µl of a 1% pepsin solution. Digestion was carried out at 37°C for 24 hr followed by a second addition of pepsin and a further incubation at 37°C until the samples were completely digested. HPLC analysis of the resultant lysates was carried out using an acetonitrile gradient in 0.1% TFA, measuring absorbance at 214 nm.

Trypsin digestion of the microcapsules and albumin was performed on samples initially treated with guanidine-HCl, DTT and iodoacetamide, to open the protein structure. 0.2% trypsin was added to these pre-treated samples and incubated at 37°C until completely digested (additional trypsin was added if required). HPLC analysis of the lysates was carried as detailed above.

The HPLC analyses showed no significant differences between the microcapsule and albumin structures. This confirms that there is significant retention of secondary and tertiary protein structure after microcapsule insolubilisation.

Coupling to FITC

FITC (fluorescein isothiocyanate) binds covalently to amino groups on the microcapsules and exemplifies the principle of derivatising charged groups, namely lysine residues, with drugs which are subsequently released by degradation of the microcapsule matrix itself.

FITC was covalently bound to three microcapsule batches. A ratio of microcapsules to FITC of 15:1 was used. 12.5 mg FITC was added to the suspended microcapsules and the mixture was incubated at 30°C for 30 minutes. Excess fluorescein was removed by washing the microcapsules until no fluorescein was present in the washing, i.e. no leaching of the marker was observed.

The microcapsules were digested with Proteinase K at a concentration of 0.4 EU/ml. The fluorescein was released from the microcapsules as they were digested, and was measured by sampling the microcapsules suspension at various time intervals. The released FITC was separated from the microcapsules by centrifugation and quantified by measuring the absorbance at 493 nm.

The results showed that, the less heat-fixed the microcapsules, the more rapid the initial release of fluorescein. However, after 225 minutes, all samples had released greater than 90% of the fluorescein. The amount of FITC bound to the different heat-fixed microcapsules was

similar, with approximately 10 ± 0.5% mole/mole loading for all three batches.

Release rates for bound actives can thus be adjusted by "setting" the degradation rate of the microcapsules prior to the attachment of the actives.

Microcapsules of Example 1 were incubated with whole human blood for 30 minutes at 37°C to determine if the microcapsules were able to stimulate platelet activation. The concentration of microparticles was equivalent to a dose of 2000 x 10^6 particles/kg. After 30 minutes incubation, the serum was tested for effects on platelet aggregation stimulated with collagen ADP and arachidonic acid. Effects on general hemostatic mechanisms were assessed by measurement of procoagulant activity, partial thromboplastin time; prothrombin time by appearance of fragments 1+2 and fibrinopeptide A; and fibrinolytic activity by examination of euglobulin lysis time.

At this concentration, there was no evidence of any untoward effects upon the assays tested. Thus the results suggest that the microparticles are inert and hydrophilic, unlike microparticles made by an emulsion processes.

In a further test, microparticles manufactured by the method of Example 1 were sterilised by gamma irradiation by exposure to a Co^{60} source and received a dose of 25-35 Kgray. The microcapsules were reconstituted in aqueous diluent at a concentration of 1.5 x 10^9 microparticles/ml and administered to healthy male volunteers at doses ranging from 25-300 x 10^6 microparticles/kg under ethical committee approval. The microparticles were hollow and contained air which enabled their passage and persistence in the blood stream to be followed using ultrasound imaging.

Using an Acuson-128, grey scale 2D images of the right and left ventricle were acquired to monitor the circulatory life time following IV dosing. For opacification of both the left and right ventricle to occur, significant levels of microparticles must be present in the chambers. At

doses from 25 x 10^6/kg upwards, the opacification in the right and left ventricles persisted for a period of 1 hour or more, showing that significant quantities of particles remained in the circulation.

This data shows the basic microcapsule vehicle to be inert to the coaggulatory machinery in the blood, and hence ideally suited to carry therapeutics. This is completely contrary to microparticles made by emulsion processes which show rapid RES uptake and circulatory half-lives of 10 minutes or less. Furthermore, the microparticles do not require derivatisation with co-block polymers to enhance the circulatory half-life.

Example 2

This Example shows that additives can be included in the spray-drying feedstock of the microcapsule wall-forming material, such that the resultant microcapsules can be heat-fixed at a lower temperature. Additives which allow lower cross-linking (insolubilisation) temperatures of the microcapsule polymer have utility when active drugs are co-spray-dried and hence incorporated in the matrix. By using these additives, microcapsules with heat-sensitive actives can be insolubilised at advantageous lower temperatures.

To the spray-drying feedstock, 5 mg/ml tyrosine was added, and microcapsules were formed, using the method detailed in Example 1. No changes in the spray-drying conditions were required to obtain microcapsules.

The collected microcapsules were heat-fixed as before, but at a temperature of 100°C for 55 minutes, significantly lower than the normal 175°C for 55 minutes, to achieve the same cross-linking. The microcapsules produced had a mean size of 3.28 ± 0.6 μm with 90% of the mass within 2-5 μm.

Example 3

Example 1 details the production of 3 μm microcapsules. This Example shows that, by adjustment of the spray-drying conditions and the use of a secondary stage classification processing step, larger microcapsules

may be produced with excellent control over size and size distribution.

20% HSA was spray-dried under the conditions shown in Table 2. The collected microcapsules were heat-fixed at 175°C for 55 minutes, deagglomerated and then classified using an elbow jet classifier (see Table 3).

Table 2

Spray Dryer Condition	Setting
Inlet Temperature	220°C
Outlet Temperature-Initial	89.1°C
Outlet Temperature-Final	89.2°C
Atomisation Pressure	2.0 bar
Damper Setting	0.5
Feed Rate	20.1 g/min
Stock Solution	20% HSA

Table 3

Classification Conditions	Settings
Primary Air	0.6 barg
Secondary Air	2.0 barg
Venturi Air	8.0 barg

The middle classified fraction was collected and reformulated, as the classification process removes much of the excipient. The resultant free-flowing dry powder was characterised as before. The microcapsules had a mean size of 12 μm, with virtually no microcapsules below 6 μm, and 85% of the mass between 9-18 μm.

By removing particles smaller than 6 μm, systemic circulation of microcapsules, following intraarterial administration, is prevented due to capillary trapping. This has the advantage of localising the deposited drug, thereby reducing the overall amount of drug required to achieve therapeutic activity at the desired site. This is desirable, particularly in the case of cytotoxics since

systemic toxicity is the major cause of detrimental side-effects.

Antibodies were then bound to the microcapsule wall surface. A FITC IgG was used to aid the detection of the bound antibody.

To 5 mg of FITC-IgG, 35 mg of sodium periodate was added. The mixture was incubated at room temperature for 1 hour, after which 20 mg microcapsules was added. The suspension was stirred for 10 minutes and then the activated antibody was bound to the microcapsules by the addition of 30 mg sodium borohydride. The reaction was allowed to proceed for 2 hours at room temperature, after which time the microcapsules were collected and washed.

A sample of the microcapsules was reduced, releasing the light chains of the bound antibodies. The microcapsules were removed and the resultant filtrate collected. The presence of FITC-labelled antibody light chains in the filtrate was measured by the use of a fluorimeter.

The linkage of antibodies to the microcapsules may also be achieved by means of tri and tetrapeptide spacers. The peptides are covalently linked to the activated sugar ring on the antibodies using the periodate and borohydride reaction detailed above. The antibodies are then linked to the microcapsules *via* this peptide spacer using EDCI, as detailed in Example 6.

Example 4

This Example shows that the incorporation of additives into the spray-drying feedstock, for example HSA, will alter the chemical properties of the microcapsules produced as in Example 1, such that the number of chemical linkage sites may be greatly enhanced.

Poly-lysine was incorporated into the spray-drying feedstock at a concentration of 5 mg/ml. The spray-drying procedure was carried out as detailed in Example 1. The microcapsules produced from this modified stock had a mean

size of 3.5 ± 0.3 μm with similar physical characteristics to the HSA microcapsules such as resuspension properties.

FITC was bound to the poly-lysine microcapsules as detailed in Example 1. The microcapsules were washed until no further release of the FITC was observed. The FITC was retained bound to the HSA microcapsules, showing no release in aqueous suspension. The microcapsules were digested as detailed in Example 1 and the total amount of fluorescein bound to the microcapsules measured.

The release rate of fluorescein upon digestion showed a more rapid release than the standard microcapsules. However, a total of 20 molar % fluorescein was measured bound to the microcapsules, an increase of 50% over the standard microcapsule composition.

Example 5

This Example shows that actives may be linked to the shell and their subsequent release rate governed by degradation of the microcapsule itself.

To a 10 mg/ml solution of methotrexate, 25 mg carbodiimide (EDCI) was added. The solution was stirred for 4 hours to initiate and ensure complete activation of the methotrexate. 50 mg HSA microcapsules, produced as in Example 1, were added to the activated drug and stirred for 3 hours at room temperature. The methotrexate was chemically bound to the microcapsules *via* the amine groups on the albumin. The microcapsules were collected and washed to remove any loosely-bound methotrexate.

The methotrexate-linked microcapsules were characterised and also analysed for drug content. The microcapsules had a mean size of 3.2 ± 0.6 μm with 90% by mass between 2-5 μm. The analysis of the drug content showed that the microcapsules did not release the drug when resuspended in an aqueous medium. Moreover, a three month stability trial showed no drug release after this time. Proteinase K digestion of the albumin microcapsules released the bound drug which was shown to be linked to only a limited number of amino acids and small peptides.

Example 6

Doxorubicin was conjugate to microcapsules produced using the general method described in Example 1, with carbodiimide. 3 mg doxorubicin, 6 mg EDCI and 100 mg microcapsules were suspended in distilled water at pH 6.6 and 37°C for 20 hr with continuous stirring and resuspension and formulated.

The doxorubicin-microcapsules were analysed for drug content and characterised. The results showed no change had occurred to the characteristics of the microcapsules during drug conjugation. Drug loading was assessed on an enzyme-digested sample of microcapsules by HPLC analysis. A loading of 2% was measured and the drug was shown to be linked only to a limited number of amino-acids or small peptides.

It has been shown previously that the activity of doxorubicin bound to polymeric carriers proves beneficial in tumours showing the multidrug resistant phenotype.

Example 7

Microcapsules produced using the method of Example 1 were derivatised to carry 2-fluoro-5-deoxyuridine (FUdR). The drug was activated with succinic anhydride.

40 mg FUdR was added to 0.2M phosphate buffer at pH 8.3. To this solution 200 mg succinic anhydride was added and the reaction mixture stirred at 30°C for 2 hours, whilst maintaining the pH at 8.3 using IM NaOH. The pH of the reaction mixture was adjusted to 6.5, and 0.5 g microcapsules added. After stirring the suspension for 10 minutes, EDCI and N-hydroxysuccinimide in a ratio of 15:1 were added, and the coupling reaction allowed to proceed at room temperature. After 24 hours, the microcapsules were collected and washed, and the presence of FUdR on the microcapsules confirmed by HPLC analysis. The acid hydrolysis of the drug-linked microcapsules was carried out in an ASTED system using 1% TFA, and the subsequent release of the FUdR was monitored at 269 nm. 15% w/w FUdR was

found to be bound to the microcapsules *via* the acid hydrolysable linkage.

Cytotoxic Activity

Cytotoxic activity of the drug-microcapsule conjugates of Examples 5 to 7 was measured *in vitro*. The HSN cell line was used. This is a chemically-induced rat colonic sarcoma, which produces liver tumours in an animal model with a vascular pattern similar to that seen in human colorectal liver metastases. Cell kill was measured indirectly using MTT. This is reduced in active mitochondria to formazan, a coloured metabolite. HSN cells were incubated in multi-well plates to which was added a control dose of drug or drug-microcapsule lysate. After a range of exposure times, MTT was added to the wells and the concentration of formazan measured as an indication of cell death. The assay was calibrated for this cell line against a range of known cell numbers.

All three drugs (methotrexate, 5-FUdR and doxorubicin) had similar dose response curves for the native drugs and the drug-microcapsules lysate, indicating that cytotoxic activity was similar. The maximum reduction in cell activity for all three drugs and lysates was approximately 80%. For 5-FUdR and methotrexate, the steepest part of the dose response curve was from 1 μg/ml down to 0.01 μg/ml and for doxorubicin from 0.1 μg/ml, well within serum ranges seen *in vivo*. Control lysates of underivatised microcapsules showed no cytotoxic activity.

Example 8

This Example illustrates linkage of active compound, not directly to the microcapsule shell wall but *via* a degradable spacer or linker. This enables greater control of both the linking and release of the active compound.

Using the technology detailed in WO-A-9317713 for linking drugs to soluble carriers, naproxen has been linked to microcapsules using a lactic acid spacer. To a 10 mmol suspension of L-lactic acid in dimethylformamide, 20 mmol triethylamine and 10 mmol pentamethylbenzyl (PMB) chloride

were added. The mixture was heated until a solution was formed and then held at room temperature. Excess sodium carbonate was added after incubation of the solution overnight, and the precipitated ester, L-lactic acid-PMB, was collected, washed and dried.

To a solution containing 10 nmol naproxen, L-lactic acid-PMB and 4-dimethylaminopyridine, 11 mmol solution of dicyclohexylcarbodiimide was added. The reaction mixture was stirred at 25°C and the formation of the naproxen linker monitored. On completion of the reaction, the naproxen-L-lactic acid linker was collected, washed and dried.

The PMB protecting group was removed by the reaction of the naproxen linker with anisole and trifluoroacetic acid at room temperature for 2 minutes. Excess reagent was removed under vacuum and the residue was collected and washed. Acidification of the washed residue produced naproxen-L-lactic acid, which was extracted, washed and dried under vacuum at 50°C.

The naproxen-L-lactic acid was activated by its 1:1 reaction with carbodiimide, followed by the addition of 1 mmol N-hydroxysuccinimide. The active naproxen-L-lactic acid-NHS was added to HSA microcapsules at a 5:1 ratio in a borate buffer. The resultant product was collected and dried.

The dried naproxen microcapsules were formulated, resulting in a free-flowing powder, with a microcapsule mean size of 3.5 ± 0.6 μm. 90% of the mass of the microcapsules was between 2 and 5 μm.

Analysis of the product was carried out using Capillary Zonal Electrophoresis (Beckman, UK). This showed the presence of the drug on the microcapsules. The release of the drug using esterases and subsequent analysis of the released naproxen were carried out using an ASTED system linked to a Gilson HPLC (Anachem UK). The drug was shown to be intact and in its native form.

Example 9

The spray-drying production of the microcapsules allows control over many facets of the process and final characteristics of the microcapsules. The surface characteristics of the final microcapsules can be altered such that ligands for enzymes or receptors may be incorporated into the microcapsule shell. In this Example, the number of arginine residues is increased, and this enhancement was used to bind TPA.

Using the method of Examples 1 and 5, poly-arginine was added to the spray-drying feedstock. Using the same conditions as described in Example 1, microcapsules are produced. The microcapsules have a mean size of 3.31 ± 0.6 μm and 90 % of the mass is between 2 - 5 μm.

To 100 mg of microcapsules, a solution containing 250 μg TPA is added. The suspension is agitated for 2 hours after which the microcapsules are removed and briefly washed. The concentration of TPA remaining in the reaction solution is measured by RP-HPLC having reduced the peptide by incubation in 20 mM DTT at 37°C for 30 min in the presence of 8 M Urea. The analysis of the fragments is carried out using a gradient of 10-40% acetonitrile-water and 0.1% TFA over 60 minutes.

The TPA-microcapsules are analysed for the presence of TPA using the clot lysis assay. A fibrin clot is produced by combining fibrinogen, thrombin and the TPA microcapsules. Plasminogen is then added to the clot and a glass bead added to the surface to allow the assay end point, i.e. clot lysis, to be determined. The fall of the glass bead through the lysed clot shows that the TPA is both bound to the microcapsules and that it is still active.

In addition, the amount of TPA bound to the microcapsules is determined by using a modified fibrin assay. To a microtiter plate well, a thin agarose gel containing fibrinogen and thrombin is added. To the gel, 20 μl suspension of TPA-microcapsules and plasminogen are added. After 30 minutes, the plate is washed and the

reduction in the gel turbidity is determined using a microtiter plate reader at 340 nm. The concentration of TPA present on the microcapsule is determined using appropriate TPA standards. The results show that between 15 and 20 % TPA is bound to the microcapsules.

The TPA microcapsules can have utility as a deposit thrombolytic agent for administration at the time of angiography, similar to that proposed in WO-A-9408627 as a deposit echocontrast agent, the advantage being maintenance of a localised reservoir of TPA in the myocardium.

TABLE 4 AMINO ACID COMPOSITION
OF NATIVE ALBUMIN AND MICROCAPSULES

Amino Acid	HSA (n = 5)	Soluble Microcapsules (n = 5)	Insoluble Microcapsules (n = 7)
Aspartic Acid	50.188 ± 2.73	57.99 ± 4.13	59.23 ± 4.81
Glutamic Acid	79.31 ± 5.44	80.47 ± 2.26	85.70 ± 4.04
Serine	23.21 ± 1.39	23.2 ± 1.23	20.65 ± 2.44
Glycine	14.54 ± 0.71	15.33 ± 0.38	14.93 ± 107
Histidine	17.34 ± 1.06	17.65 ± 1.01	16.42 ± 0.85
Threonine	26.99 ± 1.34	27.23 ± 1.56	28.39 ± 2.38
Alanine	63.83 ± 1.34	61.45 ± 0.96	62.69 ±1.86
Arginine	25.18 ± 0.95	24.39 ± 1.02	23.15 ± 1.55
Proline	28.36 ± 1.17	27.45 ± 2.03	25.20 ± 3.05
Tyrosine	18.37 ± 0.71	18.02 ± 0.42	16.13 ± 1.38
Valine	35.54 ± 1.49	35.07 ± 1.03	37.59 ± 4.53
Methionine	7.99 ± 0.5	7.68 ± 0.42	6.24 ± 1.16
Isoleucine	6.42 ± 0.54	6.64 ± 0.57	8.96 ± 1.78
Leucine	63.22 ± 3.48	62.13 ± 3.14	59.28 ± 4.38
Phenylalanine	32.00 ± 2.73	30.45 ± 2.13	30.48 ± 1.92
Lysine	56.185 ± 2.50	53.45 ± 1.62	51.68 ± 3.67

CLAIMS

1. A sterile powder comprising smooth, spherical microparticles, 0.1 to 50 μm in diameter, of cross-linked materials, the microparticles being hydrophilic and capable of reconstitution in water to give a mono-disperse suspension, and which additionally comprises a physiologically or diagnostically-active component linked directly or indirectly to microparticles *via* free functional groups thereon.

2. A powder according to claim 1, wherein the material is an amino-acid, a polyamino-acid or other polypeptide.

3. A powder according to claim 1 or claim 2, which incorporates an additional water-soluble material that facilitates enzymatic biodegradation.

4. A powder according to any preceding claim, wherein the active component is a drug, chemical spacer, a ligand for an enzyme or receptor, or an antibody.

5. A powder according to any preceding claim, obtainable from said material, which is water-soluble and has said groups, by (i) spray-drying and cross-linking, such that said groups are retained in free form, and (ii) linking the active component to the cross-linked material *via* said groups.

6. A powder according to claim 5, wherein step (i) comprises spray-drying a solution of the water-soluble material, and cross-linking the dried material with heat and in the presence of less than 4% moisture.

7. A powder according to any preceding claim, wherein the functional groups are amine, hydroxyl, carboxyl or sulfhydryl.

8. A powder according to claim 7, wherein the functional groups are amine, hydroxyl or carboxyl.

9. A powder according to any preceding claim, wherein the particles have a ratio of interquartile range to mean diameter of 0.2 to 0.5.

10. A powder according to claim 9, wherein 95% of the particles are smaller than 6 μm, and 80% of the particles are in the range of 1 to 6 μm.

11. A powder according to claim 9, wherein 90% of the particles are smaller than 20 μm, and less than 5% by volume are smaller than 6 μm.

12. A suspension of microparticles as defined in any preceding claim, suitable for parenteral administration.

13. A suspension according to claim 12, wherein the microparticles are according to claim 10, for intravenous administration.

14. A suspension according to claim 12, wherein the microparticles are according to claim 11, for intraarterial administration.

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(57) Abstract

A sterile powder comprising microparticles, 0.1 to 50 μm in diameter, obtainable by spray-drying and cross-linking a water-soluble material having free functional groups, is characterised in that the microparticles are hydrophilic, can be reconstituted in water to give a monodisperse suspension, and have retained said groups available for derivatisation. The particles are linked to drugs or other functional molecules, and used as vehicles in therapy.

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 08627 (DELTA BIOTECHNOLOGY LIMITED) 28 April 1994 see the whole document ---	1-14
A	EP,A,0 494 417 (BASF CORPORATION) 15 July 1992 see the whole document -----	1-14

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information on patent family members

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